

## Cardamonin Attenuates Tamoxifen Induced Hepatotoxicity in Rats through Modulation of Inflammatory Mediators, Oxidative Stress and Apoptosis

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### A B S T R A C T

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Tamoxifen (TAM) is an anti-estrogen used in the prevention and treatment of hormone dependent breast cancer. TAM therapy may cause hepatic injury, which may indicate the stoppage of the drug. Therefore the present study was done to clarify the possible protective role of cardamonin (CAR) against TAM induced hepatotoxicity. In this work, the protective effects of CAR against TAM hepatotoxicity were studied in female rats. Sex groups of rats were used each formed of eight rats. Group I (control group): received normal 0.5% carboxymethylcellulose vehicle orally for 7 weeks. Group II (CAR group): received CAR (30 mg/kg), orally suspended in 0.5% carboxymethylcellulose vehicle for 7 weeks. Group III (TAM 0.6 mg group): received tamoxifen in a dose of (0.6 mg /Kg/ day) for 6 weeks. Group IV: (TAM 0.6 mg & CAR group): TAM in a dose of (0.6mg /Kg/ day) orally for 6 weeks and CAR (30 mg/kg) orally, for 7 weeks. Group IV (TAM 45mg group): received TAM in a dose of (45 mg /Kg/ day) i.p., for 7 successive days. Groups VI (TAM 45mg& CAR group): TAM in a dose of (45 mg /Kg/ day) i.p., for 7 successive days and CAR (30 mg/kg) orally, for 2 weeks. TAM intoxication caused elevation of serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, malondyde(MDA) and tumor necrosis factor –  $\alpha$  (TNF  $\alpha$ ) as well as depletion of reduced glutathione(GSH) and superoxide dismutase (SOD)with degeneration and necrosis of the hepatocytes there was also decrease in serum concentrations of total cholesterol, HDL cholesterol , LDL cholesterol and triglyceride concentration compared to control group. Histopathological study revealed that CAR treatment resulted in marked improvement in histopathological and immunohistochemical pictures. Furthermore CAR treatment improves all biochemical markers compared TAM intoxicated group. In Conclusion, CAR supplementation appeared to be beneficial to a great extent in treating TAM hepatotoxicity.

**Key Words:** Tamoxifen, cardamonin, steatosis, hepatotoxicity.

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### 1. INTRODUCTION

Cancer breast is one of the most common invasive cancers in females; it affects nearly 12% of women around the world (McGuire et al., 2015).

There are many risk factors for developing breast cancer as obesity, being female, sedentary life (Kuy et al., 2016), alcohol intake, hormone replacement therapy, menopause, ionizing radiation, early first menstruation, old age, although breast cancer can also affects young females, about 11% of all new cases of cancer breast in the United States occurs in women younger than 45 years old and finally another important risk factor is the positive family history as about 5–10% of cases are due to inherited genes from the parents (Colditz et al., 2013).

Many of these risk factors are present in our community making our females at a great risk for developing breast cancer so there is an increasing need to decrease side effects of anticancer chemotherapy, which

may treat cancer and kill the patient especially with increasing incidence of cancer in young females.

CAR is a naturally occurring chalcone one of the flavonoid family which is often responsible for the plants yellow pigmentation. It is known to have many pharmacological activities as antineoplastic actions as reported in the study done by Niu et al. (2015) that demonstrated the inhibitory action of CAR on metastasis of lewis lung carcinoma cells and Park et al. (2013) who stated that CAR suppresses the colon cancer cells proliferation of by promoting the degradation of  $\beta$ -catenin, it also has vasorelaxant, and anti-platelet aggregation actions (Wang et al., 2001). It has inhibitory action on release of nitric oxide and inducible nitric oxide synthase expression as the studies done by Israf et al. (2007); Kim et al. (2010) ; Mahboub . (2016); Takahashi et al. (2011) and Chow et al. (2012) who explained the anti-inflammatory activity of CAR by studying its effect over the signaling pathway of the nuclear factor- $\kappa$ B (NF- $\kappa$ B) a protein complex controls DNA transcription and has an important role in

regulating immune system responses to infection (Yadav et al., 2011). Also the protective action of CAR in acute lung injury and in sepsis was demonstrated by Wei et al., 2012. Their results showed that CAR decreases systemic inflammatory responses, during sepsis, by the down regulation of TNF- $\alpha$  and interleukins (IL-1 $\beta$  and IL-6). Furthermore, CAR also proved to be cytotoxic toward amastigotes of amazonensis and Leishmaniasis (Ruiz et al., 2011).

In spite of well-established anti-inflammatory activity, the role of CAR as a hepatoprotective has not been yet investigated. So this study was done to determine whether CAR has a protective effect against TAM hepatotoxicity and also to study some possible mechanisms underlying its action.

Cancer breast is about 100 times more common in women than in men (Colditz et al., 2013), so this study was done on female rats.

So this study was planned to investigate possible protective role of CAR against hepatotoxicity of TAM which is one of its serious side effects. TAM a commonly used in treatment and prevention of cancer breast. TAM may be used to prevent breast cancer in those who are at high risk of developing it as females with positive family history; also it is used in those who have been diagnosed with breast cancer (Kuy et al., 2016).

## 2. MATERIALS AND METHODS

### 2.1. Materials

#### 2.1.1 Drugs and chemicals

Cardamonin powder from Sigma-Aldrich (St. Louis, MO, USA). It was suspended in 0.5% carboxymethylcellulose vehicle. Tamoxifen Citrate powder from Astrazeneca (6th of October, Giza, Egypt). TNF- $\alpha$  ELISA kit (Raybiotech, Inc., Norcross, Georgia, USA)

All drugs were prepared freshly before use. All the chemicals and drugs were of the best analytical grade.

#### 2.1.2 Animals

Forty eight adult female Sprague- Dawley rats (at 10–12 weeks old) weighing 150–170 g were used in this study after 7 days of acclimatization. The animals were purchased from Helwan farm (Holding Company for Biological Products and Vaccines; VACSERA, Giza, Egypt). The animals were kept in the animal breeding facility of the Faculty of Medicine, Benha University, Egypt, and allowed to have normal rodent diet which was supplied under hygienic conditions. Animals were

separated eight per cage at room temperature (23–27°C) with normal light/dark cycle, regular diet, and water access. Animal experiments were done according to the guidelines of the Faculty of Medicine, Benha University, Egypt, for the Use and Care of Laboratory Animals. Experimental procedures were approved by the Ethics Committee for Animal Experimentation.

## 2.2 Methods

### 2.2.1 Experimental protocol

The rats were divided into 6 groups each of them consists of 8 animals. They were caged (8 / cage).

#### Induction of TAM Hepatotoxicity:

Two doses of TAM was given first small dose 0.6 mg /Kg/ day orally corresponds to human dose for 6 weeks after Paget and Barnes (1964) and the other intraperitoneal injection (i.p.) of TAM in a dose of (45 mg /Kg/ day) dissolved in 1 ml saline for 7 successive days (Mahboub , 2016).

#### CAR treatment

The animals received CAR (30 mg/kg) orally. It was suspended in 0.5% carboxymethylcellulose vehicle (El-Naga, 2014).

#### Animal groups

The animals were randomly divided into 6 groups (consisted of 8 rats each) as follow:

**Group I:** (control group): received 0.5% carboxymethyl cellulose vehicle orally for 5 weeks.

**Group II:** (CAR group): the rats in this group received CAR (30 mg/kg), orally suspended in 0.5% carboxymethylcellulose vehicle for 7 weeks.

**Group III:** (TAM 0.6 mg group): the rats in this group received TAM in a dose of (0.6 mg /Kg/ day) for 6 weeks.

**Group IV:** (TAM 0.6 mg & CAR group): the rats of this group received TAM in a dose of (0.6mg /Kg/ day) orally for 6 weeks and CAR (30 mg/kg) orally, for 7 weeks starting one week before TAM treatment

**Group IV:** (TAM 45mg group): the rats in this group received TAM in a dose of (45 mg /Kg/ day) i.p., for 7 successive days.

**Groups VI:** (TAM 45mg& CAR group): TAM in a dose of (45 mg /Kg/ day) i.p., for 7 successive days and

CAR (30 mg/kg) orally, for 2 weeks starting one week before TAM treatment.

### **2.2.2. Sample collection**

One day after last drug dose blood samples were collected from the retro bulbar sinus of rat's eye. Blood samples were delivered in clean, dry test tubes and allowed to clot at room temperature. The serum was separated by centrifugation at 2000 rounds/ minute.

Then all rats were sacrificed under thiopental sodium (5 mg/kg) anesthesia and livers were collected and dissected into 2 longitudinal halves, the first one was placed into formalin 10% for histopathological and immunohistochemical studies and the second one was rapidly frozen and stored at liquid nitrogen  $-80^{\circ}\text{C}$  for measurement of biochemical markers.

### **2.2.3. Determination of liver function tests**

By estimating liver enzymes; ALT, AST, and Alkaline phosphatase. These markers were measured by colorimetric method through specific kits according to manufacturer instructions. Kits were purchased from Diamond Diagnostics, Egypt

### **2.2.4. Determination of lipid profile; total cholesterol, HDL, LDL, and Triglyceride serum concentration**

These markers were measured by colorimetric method through specific kits according to manufacturer instructions. Kits were purchased from Diamond Diagnostics, Egypt

### **2.2.5. Determination of lipid peroxidation marker (MDA) and antioxidants (GSH and SOD) in liver tissues**

About 100 mg of liver tissues were homogenized then centrifuged for 15 min at  $4^{\circ}\text{C}$ . The supernatant was kept at  $-20^{\circ}\text{C}$  until it was used for analysis of malondialdehyde (MDA), reduced glutathione (GSH), and superoxide dismutase (SOD) in the supernatant of liver homogenates. They were measured using a colorimetric method according to the manufacturer's instructions (Bio-Diagnostics, Dokki, and Giza, Egypt). Total protein concentration of supernatant was measured using colorimetric method of Lowery et al (1952).

### **2.2.6. Assessment of inflammatory markers**

TNF $\alpha$  in liver homogenate supernatant by using Ray BioR Rat TNF- alpha ELISA kit (RayBiotech, Inc., Norcross, Georgia, USA, Cat. No. ELR-TNF alpha-001 C).

### **2.2.7. Light microscopic study**

The rats were scarified under thiopental sodium anesthesia (5mg/kg), the abdomen was opened the liver was cut, the surrounding tissues were removed and then fixed in 10 % phosphate buffered formalin embedded in paraffin, 4–5mm thick sections were prepared stained with hematoxylin and eosin according to Dury and Wallington, 1967 and examined by light microscope.

### **2.2.8. Immunohistochemical study of apoptotic marker (caspase-3)**

Tissue specimens were fixed in formalin and embedded in paraffin according to known histological procedures. For immunohistochemical analyses, 5  $\mu$  thick sections were used for the activated Caspase-3 detection system (Biovision activated Caspase-3 [1:100]). In brief, the deparaffinization procedure was accomplished in Xylene for 1 h. Rehydration was done in 100%, 95%, 80% and 70% alcohol series for 2 min each. After immersion in distilled water for 5 min, sections were washed in PBS for 10 min and exposed to microwave radiation at 500 W for 10 min in citrate buffer (10 mM, pH 6.0) for antigen retrieval. Then the primary antibody was applied in an incubator at  $4^{\circ}\text{C}$  overnight then washed with PBS. Then, the biotinylated secondary antibody was applied then washed with PBS before incubating with the enzyme conjugate and 3-diaminobenzidine tetrahydrochloride. The whole procedure was finished after staining the sections with Mayer's hematoxylin then the sections were examined by light microscope.

### **2.2.9. Statistical analysis**

All the data are presented as mean  $\pm$  standard deviation (SD). Evaluation of differences between groups was performed using one-way ANOVA with post hoc test (LSD) between groups with SPSS 19.0 software. p value  $< 0.05$  was considered significant (Howell, 1995).

### 3. RESULTS

#### 3.1. Effect of CAR on liver enzymes in all studied group (mean $\pm$ SD).

**Table (1): Effect of CAR (30 mg/kg), TAM (0.6 mg /kg and 45 mg /kg) either singly or in combination on liver enzymes in normal adult female rats (n=8)**

Parameter	Group					
	Control	CAR	TAM 0.6mg	TAM 0.6mg + CAR	TAM 45mg.	TAM 45 mg + CAR
alanine Transaminase (U/L)	20.5 $\pm$ 1.34	21 $\pm$ 1.16	56 $\pm$ 2.4a	32 $\pm$ 1.5 a b	76.7 $\pm$ 2.47 a	38 $\pm$ 1.92 a b
aspartate transaminase (U/L)	46 $\pm$ 2.39	43 $\pm$ 2.79	85 $\pm$ 1.6a	55 $\pm$ .5 a b	99.7 $\pm$ 3.5 a	58 $\pm$ 3.8 a b
alkaline phosphatase (U/L)	154.3 $\pm$ 14.3	151.55 $\pm$ 3.44	287 $\pm$ 5.6 a	203.5 $\pm$ 7.5 a b	395.6 $\pm$ 17.7 a	246.3 $\pm$ 4.6 a b

a Significant difference as compared to control group ( $P < 0.05$ ).

b Significant difference as compared to tamoxifen group ( $P < 0.05$ ).

Table 1 showed that TAM in both doses caused significant increase in serum ALT, AST and alkaline phosphatase compared to control group ( $p < 0.05$ ). While administration of CAR caused significant decrease in serum ALT, AST and alkaline phosphatase compared to TAM group ( $p < 0.05$ ). Administration of CAR showed non-significant changes in enzyme levels compared to control rats.

#### 3.2. Effect of cardamonin on lipid profile in all studied group (mean $\pm$ SD).

**Table (2): Effect of CAR (30 mg/kg), TAM (0.6 mg /kg for and TAM 45 mg /kg) either singly or in combination on average (m+SD) serum concentrations of Total cholesterol, HDL , LDL, and Triglyceride concentration in normal adult female rats (n=8)**

Parameter	Group					
	Control	CAR	TAM 0.6mg	TAM 0.6mg + CAR	TAM 45mg.	TAM 45 mg + CAR
Total cholesterol concentration (mg/dL)	75.23 $\pm$ 4.6	72 $\pm$ 5.16	43 $\pm$ 3.4a	60 $\pm$ 3.5 a b	70 $\pm$ 5.5	74 $\pm$ 1.6
HDL cholesterol concentration (mg/dL)	48.5 $\pm$ 3.8	47 $\pm$ 3.5	30 $\pm$ 2a	39 $\pm$ 2.7 a b	45.7 $\pm$ 2.5	48 $\pm$ 2.5
LDL cholesterol concentration (mg/dL)	47.35 $\pm$ 3.7	49 $\pm$ 4.5	31 $\pm$ 4a	40.5 $\pm$ 3.5 a b	46 $\pm$ 3.8	45 $\pm$ 2.6
Triglyceride concentration (mg/dL)	44.9 $\pm$ 3.9	48 $\pm$ 3.9	30 $\pm$ 3.2a	38.5 $\pm$ 2.5 a b	42 $\pm$ 2.9	46 $\pm$ 4.1

a Significant difference as compared to control group ( $P < 0.05$ ).

b Significant difference as compared to tamoxifen group ( $P < 0.05$ ).

Table 2 showed that TAM 0.6mg /kg treatment caused significant decrease total cholesterol, HDL, LDL, and triglyceride concentration compared to control group ( $p < 0.05$ ). While administration of CAR in TAM intoxicated rats caused significant increase total cholesterol (mg/dL), HDL (mg/dL), LDL (mg/dL) and triglyceride concentration (mg/dL) compared to TAM groups ( $p < 0.05$ ). Administration of TAM 45 mg /kg caused no significant change in total cholesterol, HDL, LDL, and triglyceride concentration compared to control group and also administration of TAM 45 mg /kg and CAR caused no significant change in total cholesterol, HDL, LDL, and triglyceride concentration compared to TAM 45 mg /kg group. Administration of CAR alone showed non-significant changes in enzyme levels compared to control rats.

### 3.3. Effect of cardamonin on MDA, GSH and SOD in liver tissues in all studied groups (mean±SD).

**Table (3): Effect of CAR (30 mg/kg), TAM (0.6 mg /kg and TAM 45 mg /kg) either singly or in combination with CAR on average (m±-SD) concentrations of reduced glutathione, superoxide dismutase, malondyde , and tumor necrosis factor –  $\alpha$  in normal adult female rats (n=8)**

Parameter	Group					
	Control	CAR	TAM 0.6 mg	TAM 0.6mg + CAR	TAM 45mg.	TAM 45 mg + CAR
Reduced glutathione (U/L)	44.5±2.4	43.5±2.46	24.4±1.9 a	30 ± 2.5 a b	20.2±1.25 a	32.3±1.69 a b
Superoxide dismutase (U/L)	50±2.59	51.2±2.36	30±1.7 a	43 ± 2.5 a b	22.3±1.96 a	41.67±2.6 a b
Malondyde (nmol/g protein)	61.2±2.5	60.8±2.57	95± 5.4 a	75 ±3.5 a b	117.7±4.26 a	78.8±4.63 a b
Tumor necrosis factor - $\alpha$ (pg/g tissue)	6.9±.64	6.4±.55	25.4 ±1.5 a	14.6 ±.9 a b	30.5±2.26 a	15.5±1.4 a b

a Significant difference as compared to control group ( $P<0.05$ ).

b Significant difference as compared to tamoxifen group ( $P<0.05$ ).

Table 3 showed that TAM treatment with both doses caused significant increase in MDA level with significant decrease in antioxidant enzymes activity GSH, SOD compared to control group ( $p < 0.05$ ). While administration of CAR in TAM intoxicated rats caused significant decrease MDA with significant increase in GSH, SOD compared to TAM groups ( $p < 0.05$ ). Administration of CAR alone showed non-significant changes in enzyme levels compared to control rats.

### 3.4. Effect of cardamonin on inflammatory markers in all groups (mean±SD).

Table 2 showed that TAM intoxication resulted in significant ( $p < 0.05$ ) increase in TNF $\alpha$  levels in TAM groups compared to control animals. On the other hand, administration of (30 mg/kg) CAR resulted in significant decrease in TNF $\alpha$  level compared to TAM groups , while administration of CAR alone cause non-significant change of in TNF $\alpha$  level compared to control.

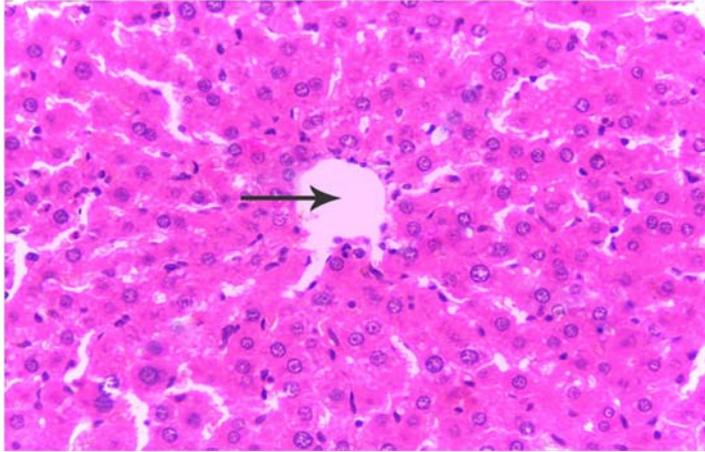
### 3.5. Effect of cardamonin on apoptosis in all groups.

Immunohistochemical study detects no expression for caspase-3 in control group. However, The TAM groups showed extensive caspase-3 expression treatment with CAR reduced expression of caspase-3 in liver sections.

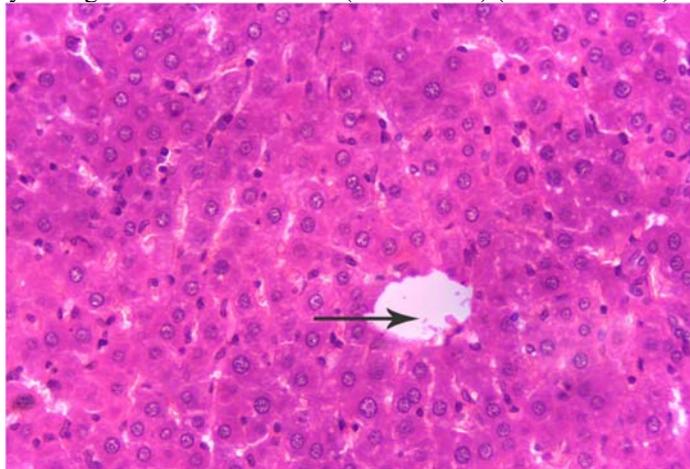
### 3.6 Light microscopic study.

Histopathological examination of liver sections revealed that the structural components of the liver in

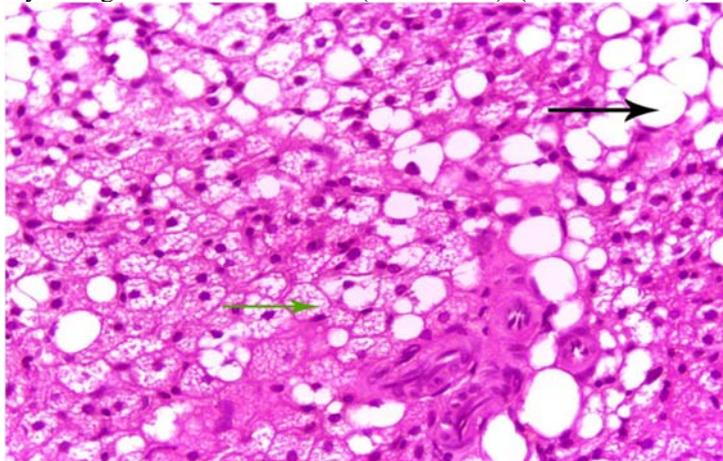
both control and CAR groups showed normal hepatic cyto-architecture. They formed of polyhedral hepatocytes which are radially arranged in anatomizing and branching plates separated by vascular blood sinusoids. The blood sinusoid is lined with endothelial all and contains many triangle kupffer cells. TAM 0.6 mg group showed marked hepatic steatosis seen as significant intracellular lipid droplet accumulation in cytoplasm of hepatocytes, but TAM 45 mg /kg intoxication, the liver sections showed marked hydropic changes, necroinflammatory foci, cellular infiltration and dilatation of blood sinusoid with diffuse Kupffer cells proliferation in between the hepatocytes and portal tract inflammation. Histopathological examination of liver sections of TAM + CRD groups demonstrated marked improvement in its histological structure in comparison to the TAM groups with less pathological changes.



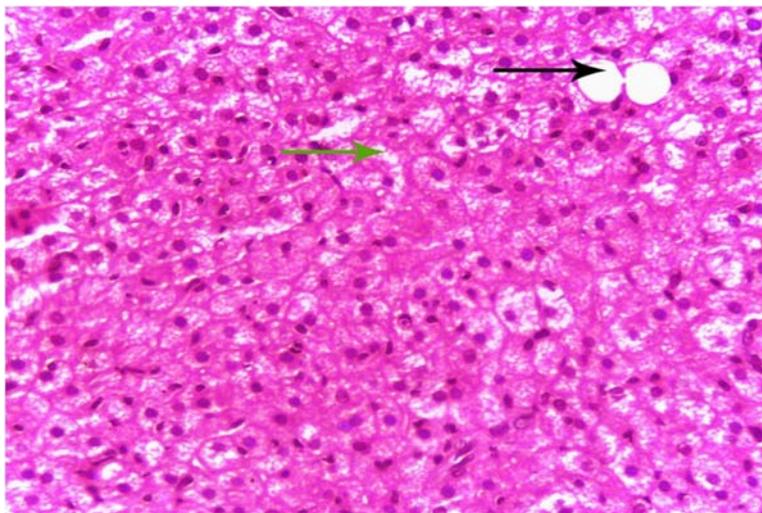
**Figure (1):** A photomicrograph of a cut section in the liver of a control rat showing normal liver architecture with normal hepatocytes radially arranged around central vein (black arrow) (H x & E x 400).



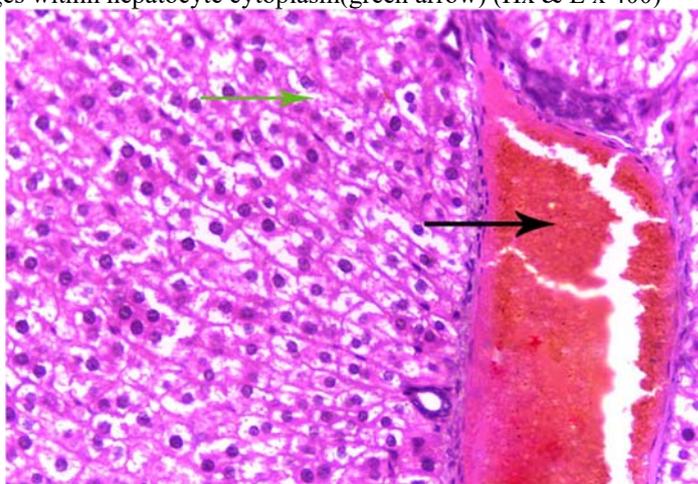
**Figure (2):** A photomicrograph of a cut section in the liver of a CAR group showing normal liver architecture with normal hepatocytes radially arranged around central vein (black arrow). (H x & E x 400).



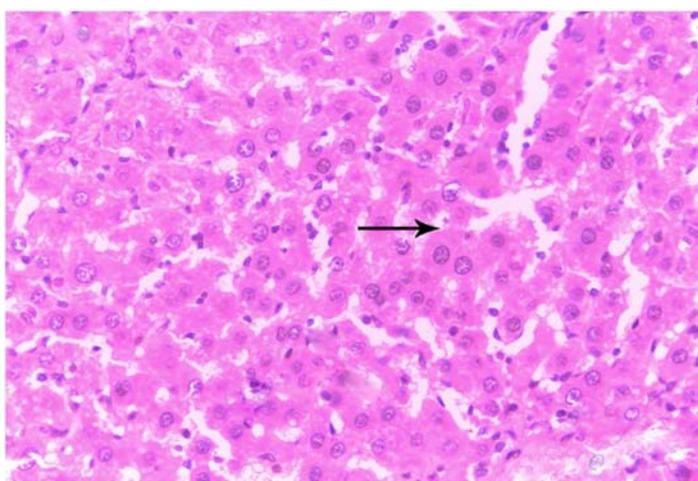
**Figure (3):** A photomicrograph of a cut section in the liver of TAM 0.6 group showing marked hepatic steatosis seen as significant intracellular lipid droplet accumulation in cytoplasm of hepatocytes macrovesicular steatosis (black arrow) and microvesicular steatosis (green arrow). (H x & E x 400)



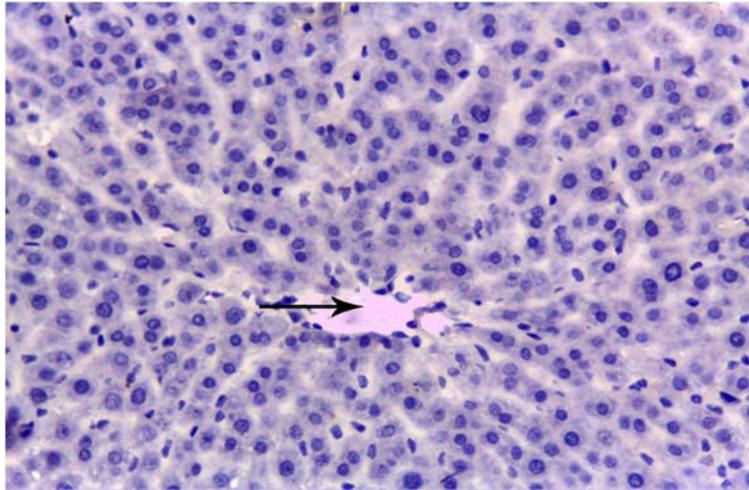
**Figure (4):** A photomicrograph of a cut section in the liver of TAM 0.6 group showing minimal hepatic steatosis seen as significant intracellular lipid droplet accumulation in cytoplasm of hepatocytes (black arrow) with moderate hydropic changes within hepatocyte cytoplasm (green arrow) (Hx & E x 400)



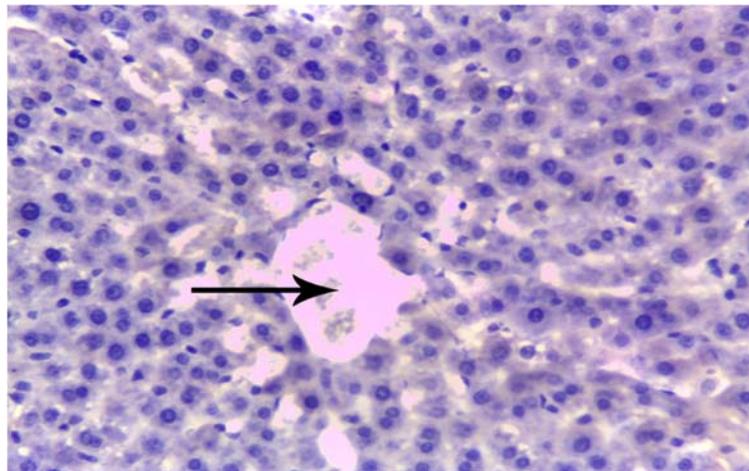
**Figure (5):** A photomicrograph of a cut section in the liver of TAM 45mg group showing preserved liver architecture, hepatocytes show marked hydropic changes (green arrow), Central vein dilated and congested (black arrow). (H x & E x 400)



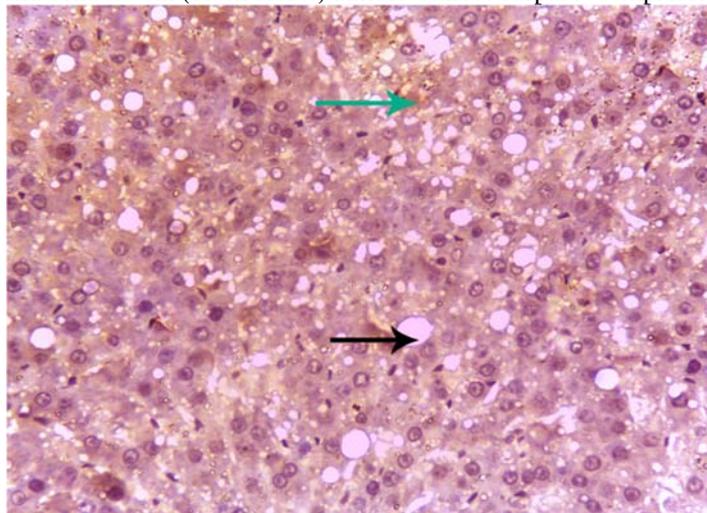
**Figure (6):** A photomicrograph of a cut section in the liver in TAM 45 mg + CAR group showing preserved liver architecture, hepatocytes show mild hydropic changes (black arrow). (H x & E x 400).



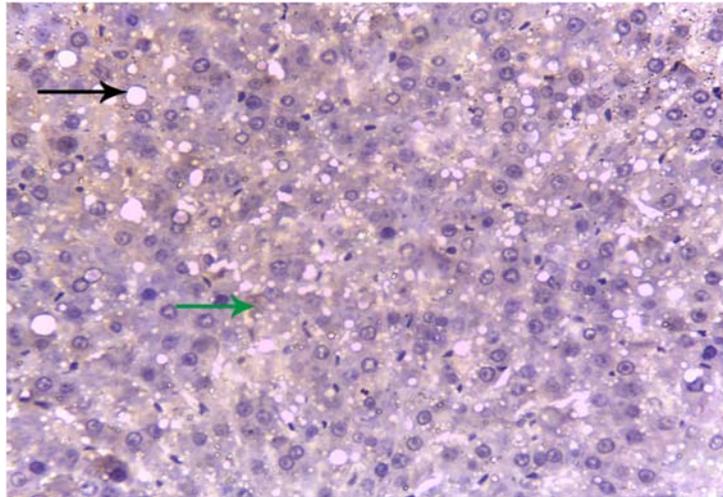
**Figure (7):** A photomicrograph of a cut section in the liver of a control group with normal hepatic architecture as radially arranged hepatocytes around central vein (black arrow) almost without caspase -3 expressions



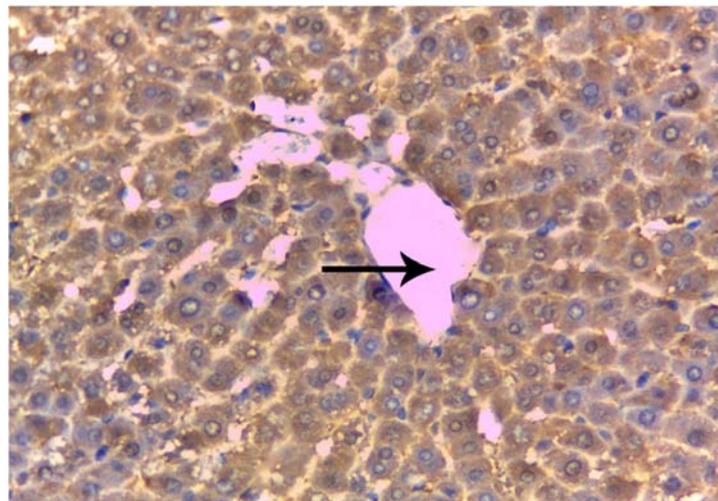
**Figure (8):** A photomicrograph of a cut section in the liver of a CAR group with normal hepatic architecture as radially arranged hepatocytes around central vein (black arrow) almost without caspase -3 expressions



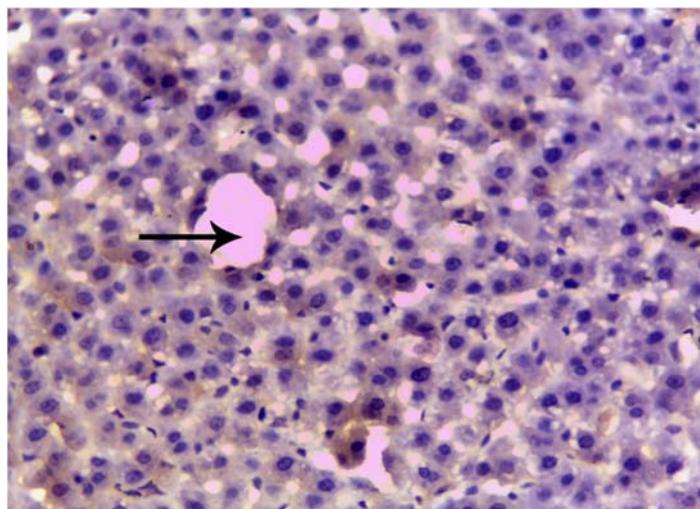
**Figure (9):** A photomicrograph of a cut section in the liver of a TAM 0,6 mg group showing marked caspase -3 expression (green arrow) with macrovesicular steatosis (black arrow).



**Figure (10):** A photomicrograph of a cut section in the liver of a TAM 0,6 mg +CAR group showing decreased caspase -3 expression(green arrow) with macrovesicular steatosis (black arrow).



**Figure (11):** A photomicrograph of a cut section in the liver of a TAM 45mg group with radially arranged hepatocytes around central vein (black arrow) showing marked caspase -3 expression



**Figure (12):** A photomicrograph of a cut section in the liver of a TAM 45mg +CAR group with radially arranged hepatocytes around central vein (black arrow) showing decreased expression of caspase -3.

## 5. DISCUSSION

Worldwide, breast cancer is one of the most common carcinomas in females. In America, breast cancer is the most prevailing female malignancy, which accounts for more than 40,000 deaths every year (DeSantis et al., 2014). Clinically, based on gene expression profiling of estrogen receptor- $\alpha$  (ER $\alpha$ ), human epidermal growth factor receptor (HER) and progesterone receptor (PR) gene, breast cancer has been classified into 4 major subtypes: Luminal A (ER/PR<sup>+</sup>, HER-2<sup>-</sup>), luminal B (ER/PR<sup>+</sup>, HER-2<sup>+</sup>), HER-2 overexpressing (ER<sup>-</sup>/PR<sup>-</sup>, HER-2<sup>+</sup>) and basal-like (ER<sup>-</sup>/PR<sup>-</sup>, HER-2<sup>-</sup>) (Moerkens et al., 2014). About two-thirds of breast cancer cases are positive for ER and/or PR, in these patient endocrine therapy with TAM or aromatase inhibitors is generally recommended as highly effective (Mauri et al., 2006). TAM is a non-steroidal anti-estrogen that is commonly used in the prevention and treatment of cancer breast. Many studies provided significant evidence that 5-year TAM therapy could improve the 5-year survival rate, particularly in postmenopausal women. Also, the initial results from the first International Cancer Breast Intervention Study-I revealed that prophylactic use of TAM reduced the risk of invasive ER-positive tumors by 31% in females who were at an increased risk for developing cancer breast (Cuzick et al., 2007).

However, the adverse effects have restricted the long-term use of TAM to a large extent. Recent studies reported that long-term use of TAM can cause many side effects such as hot flashes, night sweats, gynecological symptoms (vaginal dryness and vaginal discharge), sleep alterations, memory loss, depression, weight gain and diminished sexual function (Lorizio et al., 2012). Among all these, hepatic injury or even hepatocellular carcinoma can be one of the most serious side effects, which restricted its long-term use in many cases (Yang et al., 2013). So understanding the mechanisms involved in the development of TAM hepatotoxicity became very important as it may initiate new strategies to prevent and control TAM hepatotoxicity in the susceptible patients. Clinically, patients who accept the endocrine therapy are instructed to reexamine their liver function every 4 months due to its hepatotoxicity.

Thus, the present study was done to investigate whether TAM therapy affects the liver in chronic and acute animal study models with a dose of 0.6 mg/kg/day to investigate the chronic effects and a dose of 45 mg/kg/day TAM for 7 days treatment to build a short-term animal model also this study investigated the possible protective role of cardamonin on TAM-induced hepatotoxicity and its possible mechanism.

This study showed that TAM treatment caused significant increase in serum level of ALT and AST in both low and high doses of TAM treated groups these findings are in agree with previous reports of Fang et al.

(2016). This elevation could be due to hepatic structural damage as these enzymes are normally present in the cytoplasm of the hepatocyte and are released into circulation after cellular damage has occurred this may be due to the cytotoxic effects of TAM, also there was elevated serum concentration of alkaline phosphatase which is a specific indicator of biliary epithelium affection which may reflect compression of intrahepatic biliary canaliculi by inflammatory cells in portal tracts (McClatchey, 2011).

TAM in both low and high doses increased the oxidative stress markers in the liver as measured by the high level of MDA and significant depletion in GSH and SOD levels compared to normal control group which explains the observed leakage of cellular ALT, AST into the circulation that suggest the hepatocellular damage (Suddek, 2014).

The results showed increased levels TNF $\alpha$  which is an inflammatory marker and a potent inducer of apoptosis and cell death.

There were morphological changes at the microscopic and ultra-structural levels of hepatocytes in the TAM treated group, which was different in both groups as in the low dose of 0.6 mg/kg/day for 6 weeks there was obvious macro vacuolar steatosis characterized by large lipid vacuole within the cytoplasm of the hepatocytes. Additionally, hepatic cells in certain sections swelled to spherical shapes and fatty metamorphosis was observed, this is in agreement with numerous researches and clinical studies which have illustrated clearly that TAM causes the inhibition of mitochondrial  $\beta$ -oxidation and subsequently leads to macro vacuolar steatosis (Lee et al., 2010). The early symptoms were characterized by the presence of a large, single lipid vacuole within the cytoplasm of the hepatocytes; this is also in agreement with Labbe et al. (2008) that stated that TAM is one of the drugs that can cause macro vacuolar steatosis and steatohepatitis. This may be to the drug ability to impair mitochondrial respiratory chain thus causing not only fatty acid oxidation impairment and steatosis but also enhanced reactive oxygen species (ROS) production. ROS overproduction may be a key event in the pathogenesis of drug-induced steatohepatitis by inducing lipid peroxidation (favored by lipid accumulation), and possibly by triggering the generation of pro-apoptotic (TNF- $\alpha$ ) and pro-fibrotic (TGF- $\beta$ ) cytokines by Kupffer cells and other inflammatory cells.

In the group with a dose of 45 mg / kg TAM for 1 week the structure of the hepatic lobules became blurred, the central vein expanded and engorged. Hepatocytes appeared to undergo vacuolar degeneration there was no steatosis.

The above mentioned changes in the acute model may be explained by primary direct damage of hepatic cells

which cause hydropic degeneration and necrosis of hepatocytes. This may activate immune system to induce inflammatory changes around necrotic foci evidenced by cellular infiltration around necrotic foci and in portal tracts as well as proliferation of Kupffer cells. The marked dilatation of hepatic sinusoids may be a sign of portal hypertension secondary to pressure of necrotic and inflammatory cells on portal vein tributaries (Ferrell, 2000). The scanty fatty changes in TAM 45mg group which received TAM for only 7 days may be attributed to the fact the duration was too short to cause slowly developed fatty changes which require increase hepatic triglyceride synthesis or fat mobilization from adipose tissue to liver and fat deposition in liver (Ferrell 2000) but in low dose TAM mg group which received TAM for long duration there was obvious fatty changes in histopathology this is in agreement with the previous studies which revealed marked steatosis as (Chitturi et al., 2013; Labbe et al., 2008 ; Osman et al., 2007).

The immunohistochemical study of liver sections of both groups showed increased caspase -3 expressions compared to control group.

On the other hand, treatment with CAR ( 30 mg/kg) significantly decreased TAM hepatotoxicity as it significantly decreased liver enzymes which can indicate that CAR protected the liver cells from destruction so decreased enzyme leakage out of cytoplasm to be increased in blood, which also was obvious in the improvement in the histopathological picture and the improvement in the immunohistochemical study with decreased caspase -3 expression which is an indicator of decreased apoptosis and tissue damage in CAR group compared to TAM intoxicated groups.

Also this study found that CAR decreased TNF- $\alpha$  level which is an inflammatory marker indicating the anti-inflammatory role of CAR which is in agreement with the study done by EL-Naga. (2014).

There was also significant reduction in lipid peroxidation evidenced by the decrease in MDA level and the increase in the antioxidant enzyme activity as GSH and SOD in CAR treated groups as compared to TAM groups which can prove the antioxidant effect of CAR this is also in agreement with the study done by EL-Naga. (2014).

Furthermore, one of the major contributors of pathophysiology of TAM hepatotoxicity is the increase in hepatic cell apoptosis which could be a cause for altered epithelial integrity and cell damage, thus inhibition of apoptosis may reduce hepatic cell destruction. This is in agreement with the study done by Vince et al. (2014).

CAR (30 mg/kg) significantly down-regulates caspase-3 expression in TAM+CAR treated groups as compared to TAM intoxicated groups, proving the role of decreased apoptosis in mediating the protective role

of CAR in liver damage. This finding is consistent with El-Naga, (2014) who proved that CAR has an anti-apoptotic effect against cisplatin-induced nephrotoxicity. The anti-apoptotic action of CAR was proved by decreasing caspase -3 expression, which might be due to the suppressing effect on TNF- $\alpha$  and the oxidative stress inhibition, TNF- $\alpha$  is a potent inducer of apoptosis, activation of caspase-3, in particular, is essential for TNF-induced cell death.

This study revealed that both chronic low dose and acute high dose of TAM produced marked oxidative stress evidenced by elevation of hepatic tissue levels of prooxidant MDA and depression of natural antioxidants GSH and SOD. Moreover, tumor necrosis factor alpha was significantly increased which may reflect severe inflammation. Caspase 3 expressions were also increased which may indicate marked apoptosis. This is in agreement with Fang (2016) who showed marked DNA damage similar to what usually occur during oxidative stress in TAM treated mice. The oxidant effect of TAM is likely to be due to the ethyl group of the TAM stilbene core that is subjected to allylic oxidative activation causing DNA alkylation and strand scission. Moreover, TAM may produce widespread cellular damage through increasing cell membrane fluidity as it is highly concentrated inside lipid bilayer of cell membrane (severcan et al., 2000). Such deleterious effect was observed in a wide variety of tissues including cancer cells, hepatic cells and retina. It may be responsible for TAM induced retinopathy (Engelke et al., 2002) and liver cell damage in this experiment. It may also participate in killing of malignant breast cancer cells especially in estrogen receptor negative tumors (Boyan et al., 2003).

The marked induction of caspase -3 by TAM in this work is in agreement with the previous reports of its proapoptotic effect (lui et al., 2014). TAM promoted the apoptosis through non estrogen receptor mediated mechanism by oxidative stress. In addition, TAM may disinherit protein phosphatase 2 (Lui et al., 2014) which promotes apoptotic protein BAX (Xin and Deng, 2006) which is a strong activator of intrinsic pathway of apoptosis with consequent activation of the apoptotic enzyme cascade (caspase 3, 8) through proteolytic cleavage (Bourdeau et al., 2014).

On the other hand, TAM markedly enhanced tumor necrosis factor alpha. This is contradictory to T0-Sq et al. (2014) who demonstrated a positive feedback control between TNF $\alpha$  and estradiol. This contradiction could be solved by the fact that TAM is a pro-drug; it is activated by cytochrome p450 2D6 oxidase to the active form (4-hydroxy tamoxifen) which is a partial selective estrogen receptor modulator. It acts as estrogen antagonist in breast and as agonist in other tissues as uterus and bone (jansen and Jordan, 2003). The former effect may be responsible for inhibition of estrogen induced tumor

necrosis factor in cultured breast cancer cells observed by **TO-Sq et al. (2014)**. Contrarily in this work the same drug acts on liver cell as estrogen agonist which markedly increased tumor necrosis factor alpha.

The above mentioned studies was supported by the work of **Chuturi and Farrell (2013)** that showed that both TAM and estrogen were implicated in the pathogenesis of liver cirrhosis and hepatocellular carcinoma.

CAR pretreatment attenuated TAM induced hepatotoxicity by its antioxidant effect which was supported by the in vitro study of **Brakash et al. (2012)** who showed that methanol extract of CAR bleached carotene solution. **Kandikattu et al. (2017)** stated that the antioxidant effect of hexane extract of CAR was due to its contents of flavonoid, polyphenol, and terpenoids. He also studied the anti-inflammatory and analgesic effect of CAR evidenced by inhibition of paw edema induced by carrageenan and down-regulation of cytokines such as IL-6, COX-2, and TNF- $\alpha$  and the inhibited i-NOS mediated NO generation. Also, CAR had anti-apoptotic effect proved by caspase-3 inhibition probably due to inhibition of TNF- $\alpha$  (**EL-Naga, 2017**) which is a major inducer of the extrinsic pathway of apoptosis (**Green and Doglas, 2011**).

CAR is a strong inhibitor of lipid peroxidation which is a major cause of cell membrane damage of hepatocytes as shown by **EL-Naga. (2014)**.

There was also an interesting study done by **Fang et al. (2016)** who stated that DNA damage occurs at an early period of TAM therapy about 6 mg/kg/day TAM for 2 weeks, which in the long-run increases the risk of development of hepatocellular carcinoma, which is a serious side effect of long term TAM therapy and as CAR has been shown to be a potential anti-tumor as the study done by **Shweta et al 2017** who proved that CAR, inhibits human triple negative breast cancer cell invasiveness by down regulation of Wnt/ $\beta$ -catenin signaling cascades and reversal of epithelial-mesenchymal transition Also the study done by **Park et al ,2013** proved that CAR is effective against cancer colon by suppressing the proliferating cancer cells. CAR also inhibits the metastasis of lewis lung carcinoma cells by decreasing mTOR Activity as studied by **Pei-Guang et al 2015**. Thus the anti-tumor properties of CAR increase its promising medicinal value for patients with breast cancer due to its hepatoprotective effect and additional anticancer effect so can be combined with long-term course of TAM therapy to decrease risk of developing hepatocellular carcinoma and increasing its anticancer effect.

## 5. CONCLUSION

In conclusion, the present data showed that TAM therapy either for 6 weeks at a dose of 0.6 mg/kg/day or

for 7 days at a dose of 45 mg/kg/day caused obvious hepatotoxicity and with the fact that TAM therapy in human is recommended for 5 years liver injury will be ascertain event, this TAM induced hepatotoxicity has been attributed to estrogen receptor mediated mechanism namely TNF- $\alpha$  activation and non-estrogen receptor mediated mechanisms through oxidant and pro-apoptotic effects. So, there is an increasing need to have accurate measures for monitoring liver function and protecting it at the early stage of the TAM endocrinotherapy, prior to apparent and undesirable clinical symptoms occur.

CAR has a hepatoprotective effect which may be through the ability of CAR to reduce inflammation, oxidative stress, and apoptotic damage also it has anticancer effect which make TAM and CAR good combination in patients with cancer breast. Further studies are needed to assess the safety and the effect of CAR intake on the efficacy of TAM therapy in humans.

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