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Antitumor effect of guava leaves extract on Ehrlich Ascites carcinoma in mice *in vitro* and *in vivo*

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ABSTRACT

Background: Ehrlich Ascites carcinoma (EAC) is one of the commonest tumors that found in mice as a form spontaneous murine mammary adenocarcinoma adapted to ascites form. **Aims:** In this study we aimed to investigate the possible effect of guava leaves extract on HepG2 cells, MCF-7 cells, HCT cells, and A549 cells *in vitro*, also determination of cell cycling arrest on HepG2 cell line, and HPLC analysis of Guava leaves extract for phenolic and flavonoid compounds, and measuring the antioxidant activity of Guava leaves extract, and effect of Guava leaves extract on EAC in Swiss albino mice *in vivo*. **Material and method:** The antitumor effect was assessed by evaluating tumor volume, tumor cell count. We assessed the effect of *Guava leaves* extract on (ALT), (AST), Albumin, TP, urea, Creatinine, (CK activity), (CK-MB activity); Apoptosis was assessed by Osteopontin and caspase3 activity. **Results:** Our results indicated that Guava leaves extract have on HepG2 cell line and MCF7 cell line; also result indicated that Guava leaves extract have anti-inflammatory effect on mice by decrease liver enzyme and decrease Kidney functions and heart enzyme; Guava leaves extract also have anti-tumor effect on EAC in treated mice by inhibiting non apoptotic protein Osteopontin and increase apoptotic protein Caspase-3. **Conclusions:** Therefore this study strongly indicates that the Guava leaves extract can be considered as attractive candidates in therapeutic strategy of liver cancer and breast cancer development.

INTRODUCTION

Cancer is uncontrolled cell division which lead to formation of malignant tumor that have the ability to invade other surrounding tissue, cancer considered the second largest cause of mortality in the world **[1]**; Carcinogenesis is the formation of new malignant tumor which named by Cancer **[2]**. EAC is one of the commonest tumors that found in mice as a form spontaneous murine mammary adenocarcinoma adapted to ascites form and carried in out farmed mice by serial intraperitoneal methods, EAC considered very in important in researches as it like human tumors which are the most sensitive to chemotherapy due to the fact that they are undifferentiated and that they have a rapid

growth rate [**3**]**.** Plant extract are excellent sources for developing new medications for treating diseases, major number of drugs that are used to treat cancer are of natural origin **[4];** *Guava leaves* has a long history of traditional medicinal uses in Egypt and worldwide as a cough sedative, anti-diarrheic, management of hypertension, diabetes mellitus and in the control of obesity **[5]**, Guava extract have many component like Flavonoid and phenolic compounds, biological properties of guava have been already associated with its polyphenolic compounds, such as protocatechuic, ferulic, ascorbic, gallic and caffeic acids and quercetin **[6]**, Flavonoid and phenolic compounds have anti-inflammatory and anti-cancer effect **[7].**

2. *Materials and Methods.*

2.1 Guava Leaves (plant Extraction). The guava leaves extract was prepared according to method **[8].**

2.2 Toxicity study (LD50):

The approximate median lethal dose (**LD_{50}**) of guava leaves extract was estimated using a limited number of experimental animals. Briefly, 6 Swiss albino mice were maintained under conventional laboratory conditions (25±2°C) with free access to food**,** mice were treated with oral feeding with different doses, (**LD_{50}**) was determined according to the method **[9].**

2.3 High-performance liquid Chromatography (**HPLC**) high pressure liquid chromatograph; is a technique in analytical chemistry used to quantify, separate and identify each component in a mixture, It depends on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material; Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column **[10]**. Analysis was performed by HPLC **(Agilent 1100)** for Phenolic compounds and Flavonoid compounds

2.4 Determination of anti-oxidant activity of Guava Extract

Anti-oxidant activity of Guava Extract was measure By DPPH Radical Scavenging Activity **[11],** compared by ascorbic acid as strong anti-oxidant; by using UV-visible spectrophotometer (**Spectronic 1201, Milton Roy, USA**). All the results were performed in three replicates and averaged. The percentage inhibition (PI) of the DPPH radical was calculated according to the formula:

PI = [{(*AC*- *AT*)/ *AC*} x 100]

Where *AC* = Absorbance of the control at t = 0 min and *AT* = absorbance of the sample (DPPH) in guava extract) at t = 16 min**.**

In vitro experiments

2.5 The cytotoxicity of Guava leaves extract

The cytotoxicity of guava leaves extract on human cancer cell line (HepG2, MCF-7, HCT and A549 cell line) was carried out using Sulphorhodamine-B (SRB, Biotium, USA) dye [**12**]. Cells were seeded in 96 well microtiter plates at a concentration of 1000 cells/well. After 24 h cells were incubated for 24 h with various concentrations of guava leaves extract (0, 62, 125, 250, 500 µg/ml). After 72 h from guava leaves extract application, cells were fixed with 10 % trichloroacetic acid (150 µl/well for 1 h at 4 °C). Cells washed three times using water and stained for 10–30 min at room temperature with 0.4 % SRB dissolved in 1 % acetic acid (70 µl/well). The unbounded dye was removed with 1 % acetic acid. The plates were air dried for 24 h, the dye was solubilized with 10 mM tris base of (pH 7.4, 150 µl/well) for 5 min on a shaker at 1600 rpm. The optical density (OD) was measured spectrophotometrically at 540 nm using **(Sunrise Tecan, Elisa microplate reader, Germany)**

2.6 Effect of Guava leaves extract on Cell cycling analysis

HepG2 cells were used in cell cycling; after treatment with Guava leaves extract 52 µg/ml for 48h and non-treated HepG2 cells as positive control using ACEA NovoExpress™ software **(ACEA Biosciences Inc., San Diego, CA, USA),** according to **[10].**

In vivo experiments

2.7 The Animals and tumor model Method

1. Swiss albino mice were maintained under conventional laboratory

conditions (24±2°C) with free access to food
2. EAC is being maintained in vivo in 10–12-week-old mice by serial intraperitoneal (i.p) transplantations of 2×10^6 viable EAC cells **[13]** per animal (0.2 ml).
3. The specific dose of guava leaves extract (5000 mg/ 1 kg mice)
4. The animal was grouped to five groups:

Group A (saline): mice were orally injected with saline solution only 5 doses day after day for 10 days.

Group B (Guava extract): mice were orally injected by Guava extract only day after day with indicated extract 5 doses for 10 days

Group C (Ehrlich Ascites): mice were IP injected with Ehrlich Ascites 0.2 ml (2×10^6 cell) only.

Group D (Preventive): mice were oral injected with Guava extract two dose day after day before IP injected with Ehrlich Ascites and then injected with 3 doses of Guava extract day after day.

Group E (therapeutic): the mice were IP injected with Ehrlich Ascites Carcinoma only and then were injected with 3 doses of Guava extract day after day for 5 days.

2.8 Viability and counting EAC cells

Viability of EAC cells was determined by blue Exclusion method **[14]**. Where the total and viable cells (non-stained) were counted at magnification× 40; as the number of cells/ ml was determined in studied groups.

2.9 Biochemical parameters: the blood samples were centrifuged at 3500 rpm for 10 min to separate the serum and placed at −20°C for the biochemical determinations, including hepatic, renal and heart functions

Assessment of serum hepatic markers

Serum samples were screened for liver function tests including Albumin (ALB), Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST), Total protein (TP)) according to method **[15]** by using the fully automated **Vitros 350 chemistry System Analyzer (Ortho Clinical Diagnostic, USA)**

Assessment of renal functional markers

Serum samples were screened for kidney function tests including (urea and Creatinine) according to method **[16]** by using the fully automated **Vitros 350 chemistry System Analyzer (Ortho Clinical Diagnostic, USA).**

Assessment of heart functional markers

Serum Creatine Kinase MB (CK-MB) was measured Kinase (CK) activity was measured according to method **[15]** by using the fully automated **Vitros 350 chemistry System Analyzer (Ortho Clinical Diagnostic, USA).**

2.10 ***Apoptotic and non-apoptotic signal***

Determination of Caspase-3 activity

Caspase-3 activity determination was carried out as indicator for apoptosis; the activity of caspase-3 was determined according to **[17]** by using the colorimetric caspase-3 kit by **(sunrise- basic Tecan system, Tecan Austria Gmbh, Austria)**

Determination of Osteopontin activity

Osteopontin concentration determination was carried out as indicator for non-apoptotic signal, concentration of Osteopontin was determined according to **[18]** by the colorimetric Osteopontin kit by **(sunrise-basic Tecan system, Tecan Austria Gmbh, Austria)**

2.11 ***Histopathological examination:***

A piece of liver was fixed in 10% formalin for histopathological examination. The thin sections were cut and then stained by haematoxylin and eosin and examined under light microscope **[19]**.

2.12 ***Statistical analysis***

All statistical analyses were done by a statistical for social science package "SPSS" version 14.0 for Microsoft Windows, SPSS Inc. **[20]**. Numerical data were analyzed using the statistical package for social sciences, version 23.0 **(SPSS Inc., Chicago, Illinois, USA)**. The quantitative data were presented as mean± standard deviation and ranges when their distribution was parametric (normal).

3. Results:

3.1 ***Guava leaves extract yield***

250g of Guava leaves powder after undergoing extraction; yielded 56 g of guava leaves extract (Thick black paste).

3.2*Toxicity study:*

All doses of guava leaves extract were found to be safe up to 12000 mg extract/ kg mice, as none of the mice were dead, which suggests that guava extract may is safe compounds.

3.3 Identification of phenolic and flavonoid compounds for guava leaves extract by using HPLC:

HPLC results demonstrated phenolic and flavonoid compounds in Guava leaves extract, result of HPLC illustrated Flavonoids compounds Quercetin, Kaempferol and Luteolin (table.1); Results illustrated HPLC analysis of Phenolic compounds in guava leaves extract that contain Ellagic acid, Chlorogenic acid, Caffeic acid, Cinnamic acid and Epicatchein (Table.2);

3.4 *Antioxidant Assay:*

Guava leaves extract ability to scavenge DPPH as free radical at concentrations (5, 10, 20, 40, 80,160, 320 and 640 µg/ml), $\mathbf{IC_{50}}$ result is 18.8 µg/ml (Fig.2, A); results demonstrates Ascorbic Acid ability to scavenge DPPH at indicated concentrations (5, 10, 15, 20, 25, 30, 35 and 40 µg/ml) (Fig.2), $\mathbf{IC_{50}}$ result is 13.9 (Fig.2, B).

3.5 *Cell cycling assay*

Result of cell cycling illustrated HepG2 cell line was increased in treated cell line than control cell line in S phase from 27 to 31; also in G2 phase cell was increased from 15 to 18; Guava leaves extract have ability to make cell cycling arrest at S Phase and G2 phase in HepG2 cell line that was treated by 52 µg of guava leaves extract (table.3 and Fig.3).

3.6 Cytotoxic effect of Guava leaves extract on cancer cell lines

Results demonstrated the number of viable cells (surviving fraction) of all cell line types (HepG2, MCF-7, HCT and A549) after treatment by Guava leaves extract and determination the $\mathbf{IC_{50}}$ result 52, 97, 193, 500µg/ml respectively (Fig.1)

3.7 *Effect of guava leaves extract treatments on liver markers*

Results revealed a significant increase in AST activities from 351.8±2.3 in negative control group to 615.1±2.9 (U/L) in Ehrlich group, result indicated a significant decreased in AST activities to 375.8±2.5 and 381.5±1.6 (u/L) by (39%, 38%) in both preventive and therapeutic groups respectively, $p \leq 0.001$ (table 4 and Fig.4). Results revealed that a significant increase in ALT activities from 61.3±2.1 in negative control group to 77.6±1.2 in Ehrlich group, result indicated a significant decreased in ALT levels to 68.0± 1.2 and 70.3 ± 0.8 (u/L) by (12%, 9%) in both preventive and therapeutic groups respectively, $p \leq 0.001$ (table.4 and Fig.5). Result revealed A significant decreased in albumin concentration was observed in Ehrlich group from (2.5±0.00) (g/dL) to 1.9±0.04; result indicated a significant increase in ALB concentration to 2.2±0.05 (g/dL) and 2.0±0.05 (g/dL) by (16%, 5%) in both preventive and therapeutic groups, respectively, $p \leq 0.001$ (table.4 and Fig.6). Results revealed that a significant decreased in TP concentration from 5.5±0.07 in negative control group to 4.7±0.11 in Ehrlich group, results indicated a significant increase in TP concentration to 5.1±0.05 and 5.0±0.06 (u/L) by (9%, 6%) in both preventive and therapeutic groups respectively, $p \leq 0.001$ (table. 4and Fig.7).

3.8 *Effect of guava leaves extract on renal functional markers*

Results showed a significant increase in urea and Creatinine levels in positive control compared to negative control. The urea and Creatinine levels were found to be 72.3±1.9 (mg/dl), and 0.17± 0.02 (mg/dl) in positive control group compared to negative control groups 53±0.6 and 0.15±0.00. While treatments by guava leaves extract showed a significant decrease of urea levels to 58.0±0.89 (mg/dL) and 59.1±0.7 by (20%, 18%) in preventive and therapeutic groups, respectively; $p \leq 0.001$ (table.4 and Fig.8); also treatments by guava leaves extract showed a very highly significant decrease of Creatinine to 0.15± 0.00 (mg/dL) and 0.15± 0.00 by (12%) in preventive and therapeutic groups, respectively, $p \leq 0.001$ (table 4 and Fig.9)

3.9 *Effect of guava leaves extract treatments on cardiac functional markers*

Results revealed that a significant increase in CK-MB activities from 356.5±3.0 (U/L) in negative control group to 754.1±3.7 in Ehrlich group; Result indicated a significant decreased in CK-MB activities which found to be 374.6±2.5 and 383.5±3.0 (U/L) by (50%, 49%) in both preventive and therapeutic groups respectively, p≤0.001 (table.4 and Fig.10). Results revealed that a significant increase in CK activities from 3105.6±4.0 (U/L) in negative control group to 4360.3±3.2 in Ehrlich group; Result indicated a significant decreased in CK activities to 3106.3±3.9 and 3176.1±3.0 (u/L) by (29%, 27%) in both preventive and therapeutic groups, respectively p≤0.001 (table.4 and Fig. 11).

3.10 *Effect of guava laves extract on Ascites volume*

Results revealed Ascites fluid increased in the peritoneal cavity of the experimental animals after the inoculation of Ehrlich Ascites Carcinoma cells. The tumor bearing animals presented a marked increase in tumor volume, by treatment with guava leaves extract there is obvious decrease in the tumor volume EAC by (84.5, 71.0%) respectively in preventive and therapeutic group compared to Ehrlich group, p<0.001 (table.4 and Fig.13).

3.11 *Effect of guava laves extract on Ascites carcinoma cell count*

Cell count of ascites fluid of tumor bearing animals was found to be decreased on treatment with guava leaves extract by (27.9%, 24.2%) respectively in preventive and therapeutic group compared to Ehrlich group, p<0.001 (table.4 and Fig.12).

3.12 *Effect of treatment on apoptotic signaling (Caspase-3) and non-apoptotic signaling (Osteopontin)*

Results showed that a significant decrease in caspase-3 activities from 9.93±0.01 (pg/ml) in negative control group to 7.21±0.0 in Ehrlich group; Result indicated a significant increase in caspase-3 activities to 9.02±0.01 and 8.51±0.0 (pg/ml) by (25%, 18%) in preventive and therapeutic groups, respectively p≤0.001, (table.4 and Fig.14). Results showed that a significant increase in Osteopontin from 0.95±0.0 (pg/ml) in negative control group to 1.36±0.0(pg/ml) in Ehrlich group; Result indicated a significant decrease in Osteopontin activities to 0.55±0.01 and 0.61±0.0 (pg/ml) by (60%, 55%) in both preventive and therapeutic groups, respectively, p≤0.001 (table 4 and Fig.15).

3.14 *Histological results*

Histological studies revealed that liver from **control negative group** demonstrated normal histological features of hepatic parenchyma, with almost apparent intact hepatocytes with intact subcellular details (arrow) and intact vasculatures (star) as well as hepatic sinusoids without abnormal changes (Fig.16,A). **Guava group** showed normal histological features of hepatic parenchyma, with intact subcellular details with almost apparent intact hepatocytes (arrow) and intact vasculatures (star) as well as hepatic sinusoids without abnormal changes (Fig.16, B).

Positive control mice liver Showed diffuse lobular vacuolar degenerative changes of most of hepatocytes (black arrow); Display moderate dilatation and congestion of hepatic blood veins (star). Show Many focal areas of hepatocellular necrotic, depress infiltrated with mononuclear inflammatory cells mixed with hyperchromatic tumor cells were shown allover hepatic lobules (red arrow), (Fig.16, C)

Preventive group showed significant reduction of focal necrotic areas allover hepatic parenchyma with minimal records (red arrow), and Show Moderate records of vacuolar degenerative changes were shown (black arrow), with moderate dilated hepatic blood veins (star) compared to positive control (Fig.16, D).

Therapeutic group, exhibited moderate focal areas of hepatocellular necrotic, depress infiltrated with mononuclear inflammatory cells mixed with hyperchromatic tumor cells were shown allover hepatic lobules (red arrow); and moderate dilatation and congestion of hepatic blood veins (star) (Fig.16, E).

4. Discussion

Cancer is the most common complex disease that effect on human health in the world, more than 100 distinct types of human cancer have

been described and various tumor subtypes can be found within specific organs **[21]**; Cell line culture have fundamental role to detect the effect of new drug on cell line growth, cell line culture is safe mode in many research *in vitro*, the use of the cell model is the origin of the development and testing of anticancer drugs that presently used **[22].**

4.1Toxicity study

None of the mice were dead, which suggests that guava extract may be safe compounds our result in agreement with study **[23],** indicated the safety of guava leaves extract in herbal formulations.

The cytotoxic effect of guava leaves extract on treated HepG2 cell line, the cytotoxic effect of guava leaves extract on treated HepG2 cell line was presented by IC_{50} 52 µg/ml; the cytotoxic effect of guava leaves extract on MCF7 is presented by IC_{50} is 97.5; effect of Guava extract on HCT cell line and result of IC_{50} is 193 µg/ml ; the effect of extract on A549 cell line and cytotoxic effect on A549 with IC_{50} is 500 µg/ml; hence this result indicate that the more cytotoxic effect of guava extract have on HepG2 cell line and MCF7 cell line with low IC50 value (52 and 97.5 µg respectively).

4.1 HPLC result

HPLC result demonstrated different component of Guava extract that contain variable content of flavonoids and phenolic compounds; Phenolic compounds are a class of chemical compounds consisting of one or more hydroxyl groups (OH) bonded directly to aromatic hydrocarbon group, (table 2) illustrate HPLC analysis of Phenolic compounds in guava leaves extract that contain Ellagic, Chlorogenic acid (CGA) , Caffeic acid, Cinnamic acid and Epicatchein. CGA is component of polyphenols among the beverages analyzed in guava leaves its concentration in Guava extract was 15 mg/gm, A study about the effects of CGA in experimental animals show that therapy with CGA significantly reduced triglyceride levels in the mice liver **[24]**, CGA also exerts many biological properties, including antibacterial, antioxidant, and anti-carcinogenic activities **[25]**. **Epicatechein** is the second phenolic which present with concentration 10.3 mg/gm, Epicatechein was shown to cause DNA damage and induces apoptosis in acute myeloid leukemia cells **[26]**, Epicatechein has the ability to inhibit prostate cancer **[27]. Caffeic acid** has been reported to have a wide variety of biological activities including anti-thrombosis, antihypertensive, anti-fibrosis, antiviral, antioxidants and anti-tumor properties **[28]. Cinnamic acid** is one of phenolic compounds that found in Guava leaves extract with concentration 4.5 mg/gm, Cinnamic acid have potential functions as anti-tumor activity, it has antioxidants properties due to their strong free radical and has antimicrobial activity**[29]. Ellagic acid** is heterotetracyclic phenolic compound its concentration in Guava extract 3.25 mg/gm, Ellagic acid is an efficient multiple-function protector against oxidative stress and have vital role against colorectal cancer **[30].**

4.2 Antioxidant assay

the result clarify that different concentration of guava extract was (5, 10, 20 and 40 µg/ml) have scavenging ability was (2.2, 18.5, 54.2 and 83.01%); this result illuminate that the extract have antioxidant scavenging ability with IC_{50} is 18.8 µg/ml, the IC_{50} the number at which 50% of DPPH are removed; Ascorbic Acid ability to scavenge DPPH at indicated concentrations (5, 10, 15, 20, 25, 30, 35 and 40 µg/ml), IC_{50} value in figure 2 is 13.9 µg/ml. taken together these results (Fig.2: A,B) guava extract considered good antioxidant and have the same effect of Ascorbic Acid **[31]**.

4.3 Cell cycling

Cell cycle is stages of cell growth (G1, S, G2) and formation of two daughter cell, Cell cycle arrest is a stopping point in the cell cycle in which no longer involved in the processes surrounding duplication and division**[32]**, During cell cycle there are many check points that control in cell cycling division their position in between two phases called the G1/S checkpoint and the G2/M checkpoint at this checkpoint cell stop and no growth of cell, all check points can stop cell cycle they consider vital role to inhibit cancer progress **[33]**. Our results verify that Guava extract make cell cycle arrest of HepG2 that treated by 52µg; the result demonstrate increase number of treated cells more than control cells

at S phase and G2 phase; the results explain that Guava extract cause increase in number of HepG2 cells in S and G2 phase and it mean cell cycling arrest in cancer cell more than control cells (table 3 and Fig.3) **[34]**.

4.4 Biochemical marker

Liver functions includes Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin and total protein levels, as well as the tissue histological assay are known to be useful in assessing the functional integrity of the liver **[35];** Aminotransferases (AST and ALT) are the first enzymes to be used in diagnosis of liver damage. Since these are normally located in the cytosol, toxicity affecting the liver with subsequent breakdown in membrane of the cells leads to their spillage into plasma while their concentration rises in the blood stream **[36]**. Liver damage induced by tumor cells generally reflects disturbances in liver cell metabolism, which lead to characteristic changes in serum enzyme activities. The increased levels of serum AST and ALT may be interpreted as a result of liver damage or as changes in membrane permeability indicating the severity of hepatocellular damage by EAC. Serum liver enzymes (ALT, AST) showed significantly increase in EAC bearing mice. The increment in serum enzymatic activities is related to hepatic parenchymal damage since ALT and AST are released from mitochondrial and cytosolic localization and cellular rupture allows the enzyme to escape into the blood **[37]; Results** confirmed that a significantly increase in activities of liver enzymes (ALT & AST) activity in Ehrlich group as compared to negative control group, This increased revealed that hepatotoxicty due to cancer **[38]** and decreased albumin and total protein concentration; Treatment with guava leaves extract reduced these increased enzyme activity and recovery towards normal levels. Also, serum albumin and total protein showed significant decrease in Ehrlich group and this decrease was improved by treatment. These findings strongly proved the ability of guava leaves extract in protecting hepatocyte against membrane fragility and may stabilized the hepatic cellular membrane damage which may decreasing the leakage of enzymes into blood circulation **[39]**, **Nephrotoxicity** was proved by significant elevations in serum levels of Creatinine and BUN as compared to normal control group. The transplantation of EAC into mice group induced increase for both urea and Creatinine levels in serum that may be attributed to renal damage as a result of cancer cell invasion **[38]**. Results confirmed that a significantly increase in concentration of kidney function (Urea &Creatinine) concentration in Ehrlich group as compared to negative control group, Treatment with guava leaves extract reduced these functions and recovery towards normal levels, These findings strongly proved the ability of guava leaves extract in protecting renal toxicity **[39].**

Creatine kinase- MB (CK- MB), Creatine Kinase (CK) is predominantly found in the cytoplasm; within myocytes, the enzyme occurs in close association with the sarcoplasmic reticulum, mitochondria and myofibrils. CK-MB activity was significantly increase in positive control group compared to negative control group .This increased may be due to the excessive production of free radicals and lipid peroxides that might have caused leakage of cytosolic enzymes and cell membrane damage **[40]**. Treatment with guava leaves extract showed significantly decreased in CK and CK-MB in the studied groups.

Ascites volume and Ehrlich cells count

The Ascites volume is decreased after treatment in preventive and Therapeutic groups by 84.5%, 71% respectively (Table.4 and Fig.13); Guava leaves extract decrease Ehrlich cells counting after treatment in preventive group and therapeutic by 27.9%, 24.2% respectively (Table.4 and Fig.12).

Apoptotic and non-apoptotic

Apoptotic signal that measured by Caspase-3 activity, Result demonstrates that the guava leaves extract has the ability to increase Caspase-3 activity in preventive and therapeutic group (table 4 and Fig.14).

Non apoptotic signal is Osteopontin concentration which is a phosphor-protein expressed by neoplastic cells involved in the malignant potential and aggressive phenotypes of human malignancies **[41]**, results demonstrate that the guava leaves extract has the ability to decrease Osteopontin in preventive and therapeutic group (Table.4 and Fig.15)

Histology

The histology of liver cell is actually explaining the image of shape liver cell which indicates no changes in cell shape with any necrotic cells that appear in normal group and guava group (Fig.16, A)**. Ehrlich** group which is group of cancer cells the image clarify focal areas of hepatocellular necrotic and mononuclear inflammatory cells (Fig.16, C). **Preventive** group image demonstrates significant reduction of focal necrotic areas allover hepatic parenchyma (Fig.16, D). **Therapeutic** group image illustrate moderate reduction of focal necrotic areas allover hepatic parenchyma (Fig.16, E)**.**

This results point to the anti-tumor effect of guava extract on tumor cell was significant in preventive group and appears in therapeutic group but less than preventive; the guava extract have anti-tumor effect on liver cancer cell only compared by guava group and normal group. This present study provides new understandings about anticancer effect of Guava leaves extract *in vitro* and *in vivo* **[42];** results also illustrate that guava extract arrest cell cycle at S phase and G2 phase. The guava extract exhibit high cytotoxic effect on HepG2 and MCF7 cell line more than HCT and A549 cell line *in vitro*. also results from this study show capacity of guava extract to induce apoptotic signaling (Caspase-3) and suppress non- apoptotic signaling (OPN) in EAC mice; guava leaves extract can be used to develop a new anticancer drug in liver and breast cancer diseases.

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Table 1: HPLC result of Flavonoids compounds

Peak No.	Time (min.)	Identified Compound	Concentration(mg/gm)
1	6.80	Quercetin	25.09
2	9.21	Luteolin	3.66
3	9.55	Kaempferol	14.05

Table 2: HPLC result of phenolic compound

Peak No.	Time (min.)	Identified Compound	Concentration (mg/gm)
1	5.22	Ellagic	3.25
2	6.52	Chlorogenic acid	15.24
3	7.25	Caffeic acid	7.14
4	8.2	Cinnamic acid	4.51
5	9.01	Epicatchein	10.33

Table 3: Cell cycle result

	Control HepG2			Treated HepG2		
	Freq G1	Freq S	Freq G2	Freq G1	Freq S	Freq G2
Reading of cells %	51	28	17	43	31	19
	51	27	19	39	32	21
	55	26	15	43	32	18

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Table 4: Results of Biochemical parameters

	Control	Guava	Ehrlich	preventive	Therapeutic
AST	351.8±2.3	351.3±2.1	615.1±2.9	375.8±2.5	381.5±1.6
ALT	61.3±2.1	61.0± 1.8	77.6±1.2	68.0± 1.2	70.3 ± 0.8
ALB	2.5±0.06	2.5±0.00	1.9±0.04	2.2±0.05	2.0±0.05
TP	5.6±0.06	5.5±0.07	4.7±0.11	5.1±0.05	5.0±0.06
Urea	53±0.6	52.1±0.98	72.3±1.9	58.0±0.89	59.1±0.7
Creatinine	0.15± 0.00	0.15± 0.00	0.17± 0.02	0.15± 0.00	0.15± 0.00
CK-MB	356.5±3.0	356.5±3.0	754.1±3.7	374.6±2.5	383.5±3.0
CK	3105.6±4.0	3065.5±2.8	4360.3±3.2	3106.3±3.9	3176.1±3.0
EAC count	-	-	130.0±0.63	93.66±0.81	98.5±0.8
Ascites volume	-	-	3.5±0.05	0.55±0.05	1.03±0.05
Caspase-3	9.93±0.01	9.70±0.41	7.21±0.0	9.02±0.01	8.51±0.0
Osteopontin	0.95±0.0	0.90±0.01	1.36±0.0	0.55±0.01	0.61±0.0

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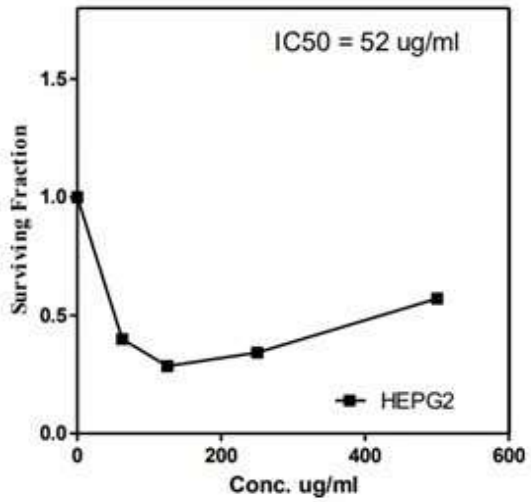
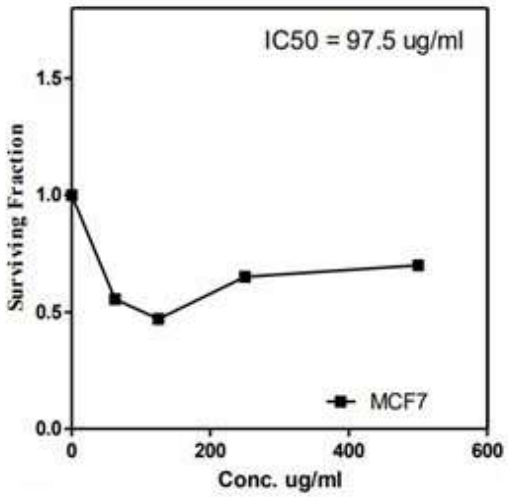
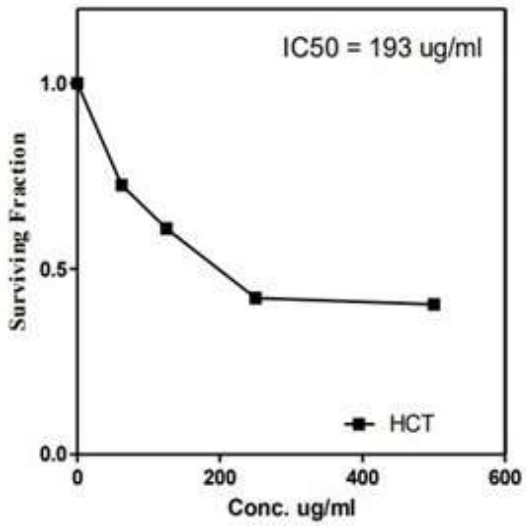


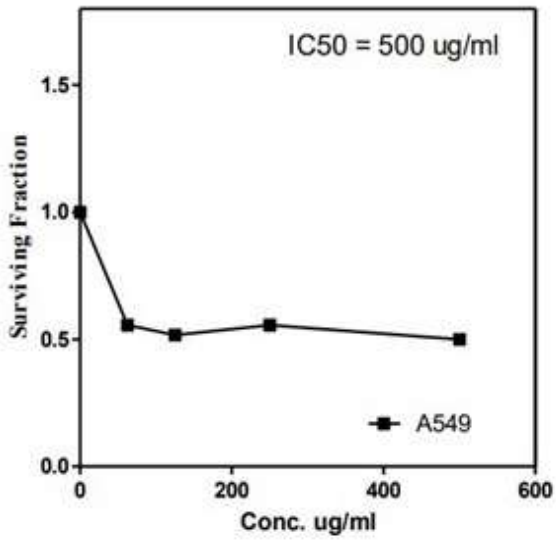
A: cytotoxicity of Guava leaves extract on HepG2 cell



B: cytotoxicity of Guava leaves extract on MCF7 cell



C: cytotoxicity of Guava leaves extract on HCT cell line



D:cytotoxicity of Guava leaves extract on A549 cell line

Figure1: cytotoxicity of guava leaves extract on Cell line

A: HepG2; B: MCF7; C: HCT; D:A549.

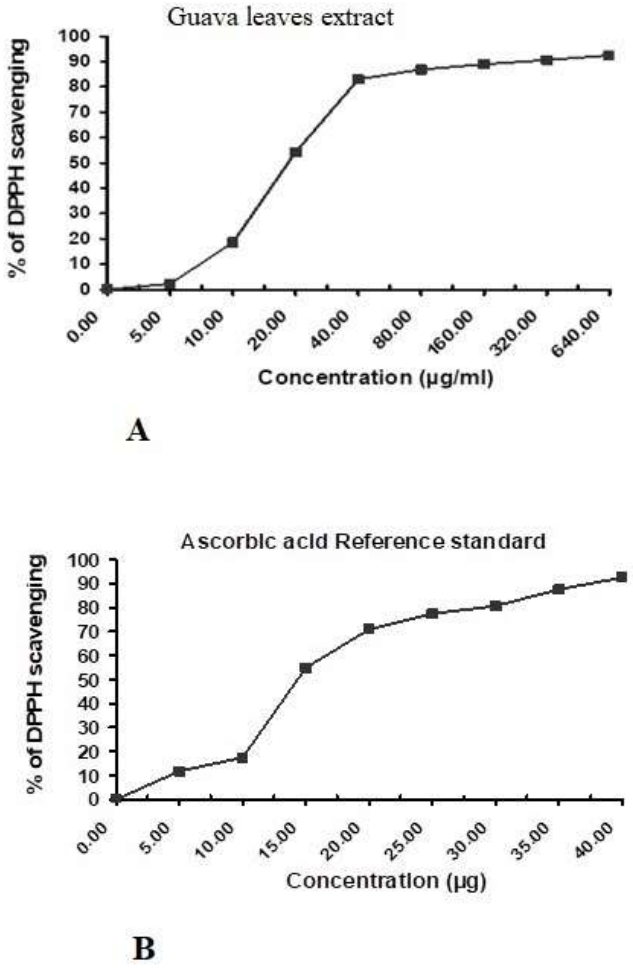


Figure 2: Scavenging ability of Guava extract and Ascorbic acid to DPPH

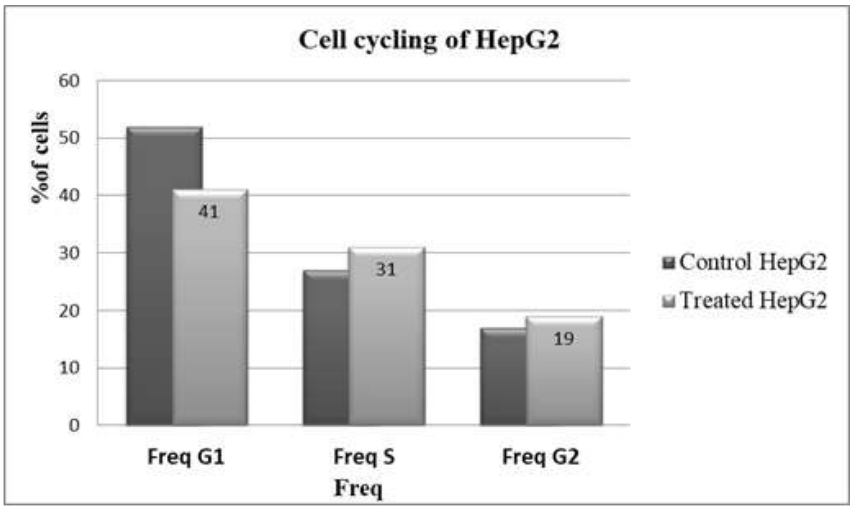


Figure 3: Cell cycling comparison between normal Control $HepG_2$ and treated $HepG_2$

A: Guava leaves extract; B: Ascorbic acid

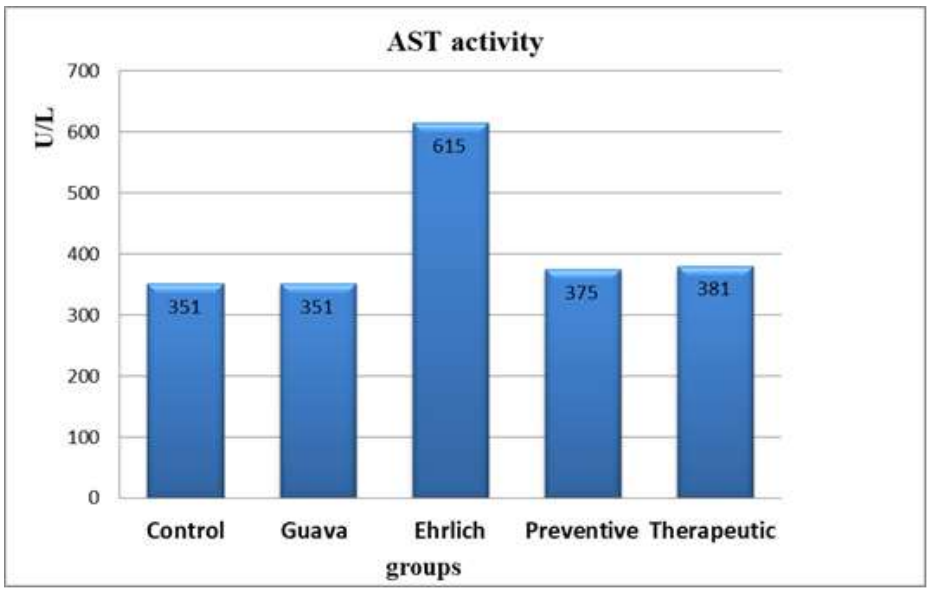


Figure 4: Effect of guava leaves extract on AST activity in all mice groups

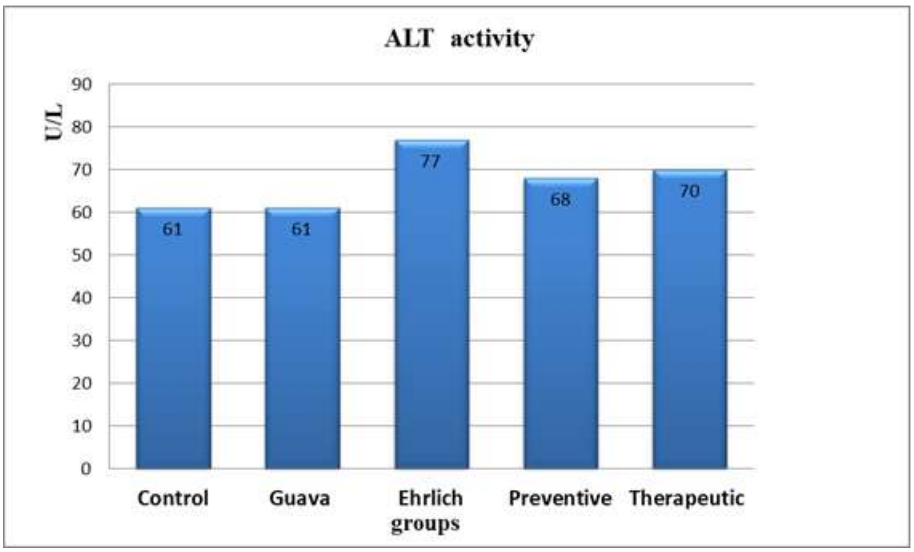


Figure 5: Effect of guava extract on ALT activity in all mice groups

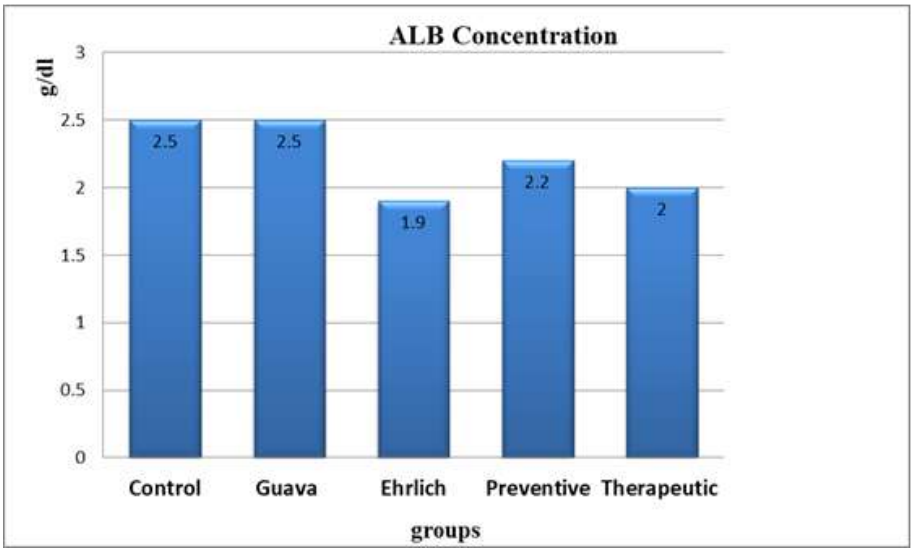


Figure 6: Effect of guava extract on ALB concentration in all mice groups

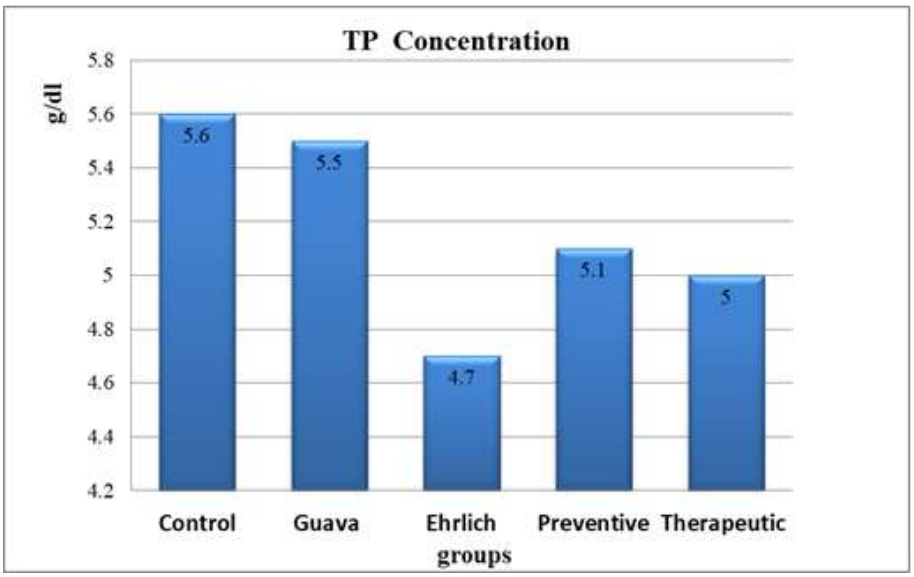


Figure 7: Effect of guava extract on TP concentration in all mice groups

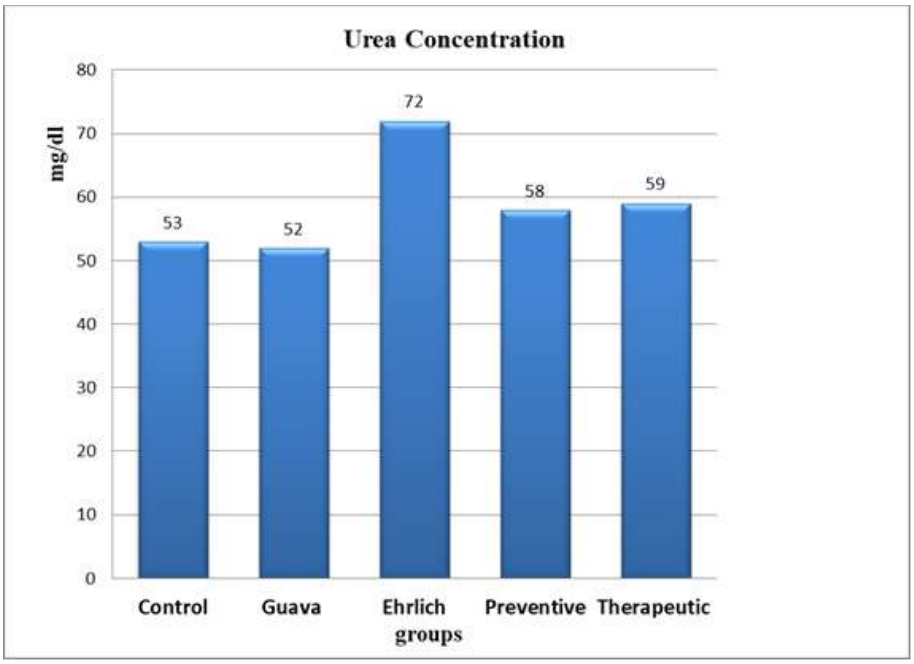


Figure 8: Effect of guava extract on Urea concentration in all mice groups

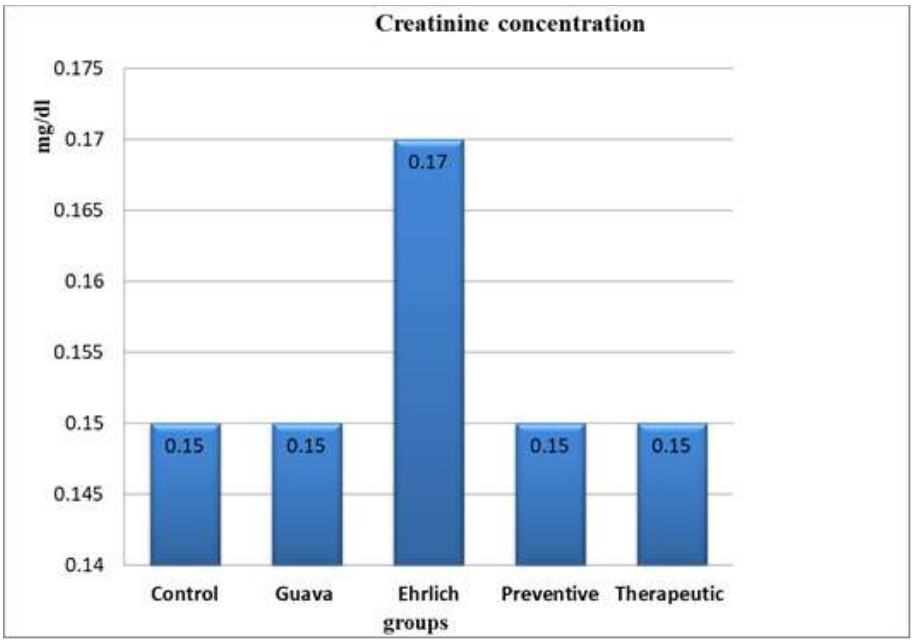


Figure 9: Effect of guava extract on Creatinine concentration in all mice groups

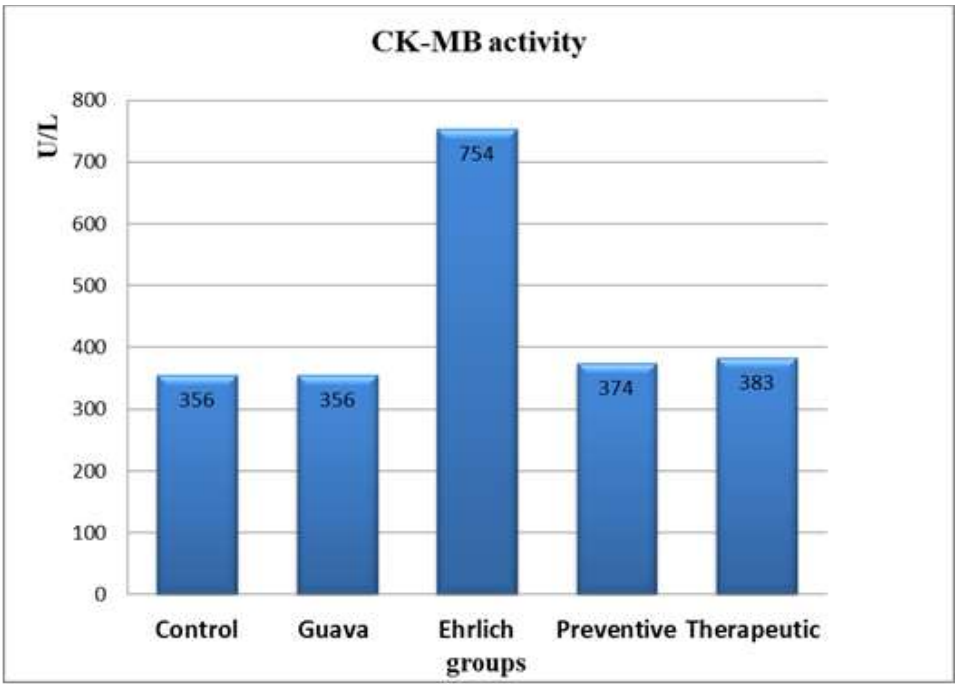


Figure 10: Effect of guava leaves extract on CK-MB activity in all mice groups

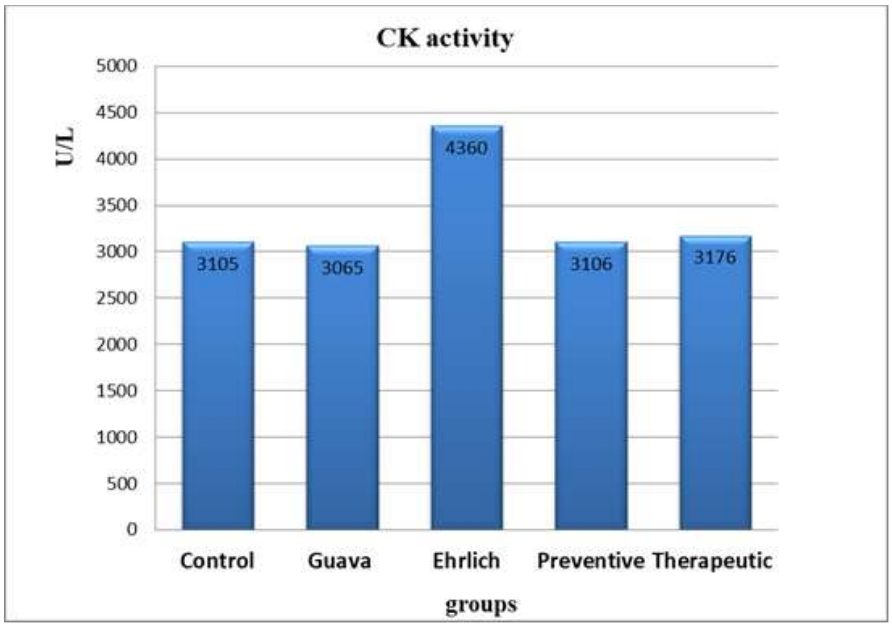


Figure 11: Effect of guava leaves extract on CK activity in all mice groups

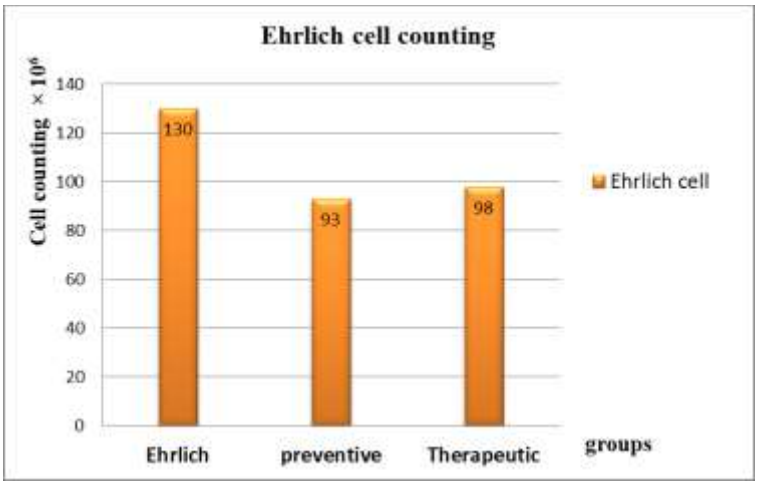


Figure 12: Effect of Guava leaves extract on Ehrlich cells counting

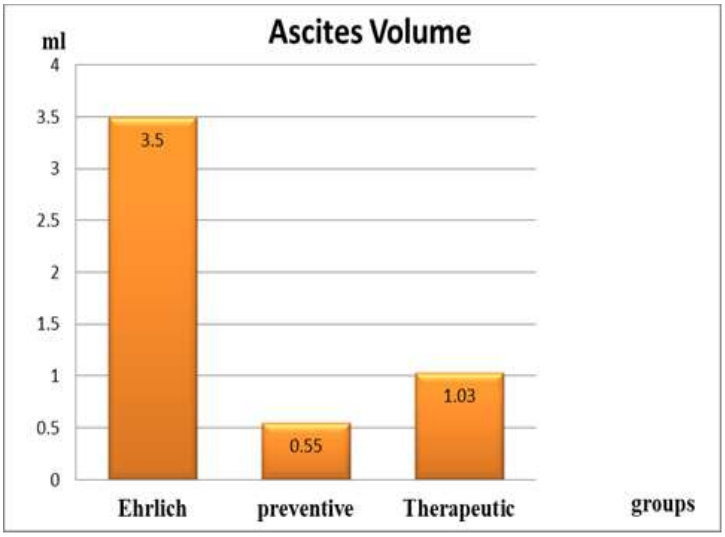


Figure 13: Effect of guava leaves extract on Ascites volume

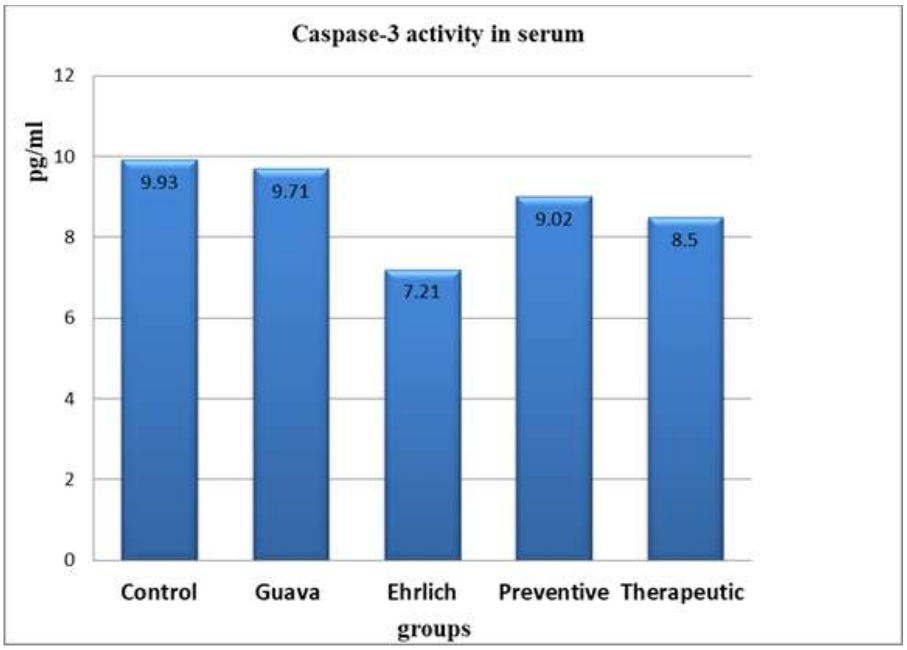


Figure 14: Effect of guava leaves extract on Caspase-3 activity in all mice groups

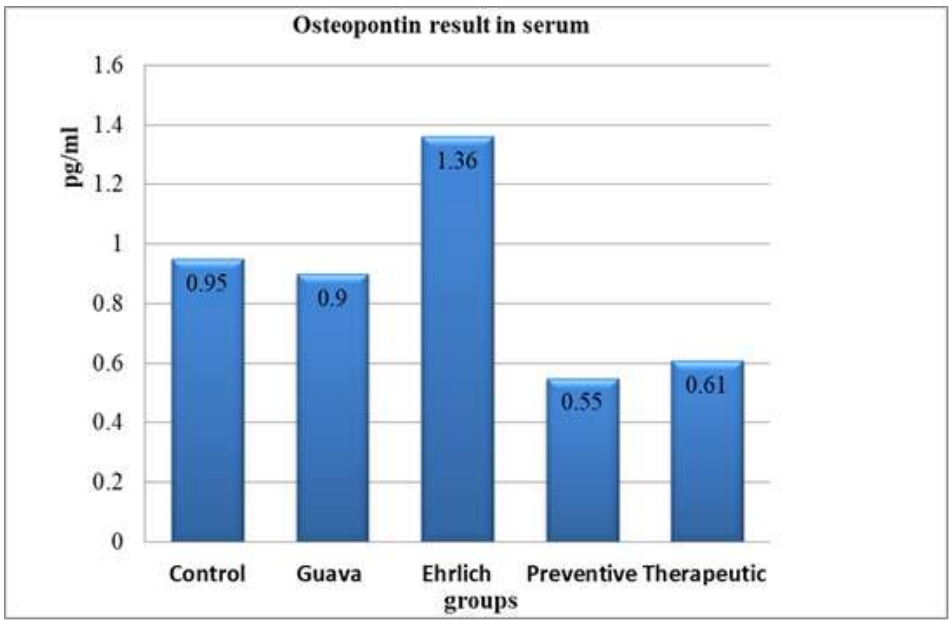


Figure 15: Effect of guava leaves extract on Osteopontin activity in all mice groups

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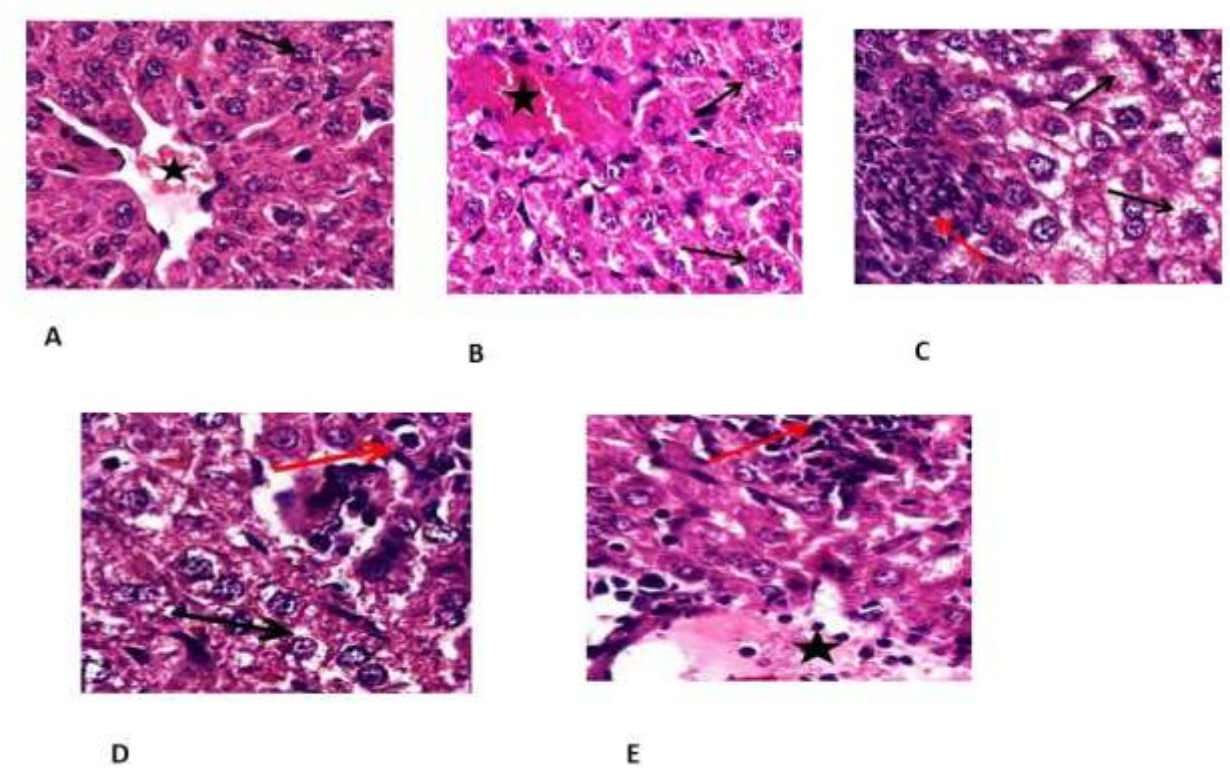

Fig.16): A): Normal group: demonstrated normal histological features of hepatic parenchyma, with almost apparent intact hepatocytes; **B): Guava group** demonstrated normal histological features of hepatic parenchyma, with almost apparent intact hepatocytes; **C): EAC group:** liver Showed diffuse lobular vacuolar degenerative changes of most of hepatocytes (black arrow); Display moderate dilatation and congestion of hepatic blood veins; **D): Preventive group:** show significant reduction of focal necrotic areas allover hepatic parenchyma with minimal records (red arrow), and Show Moderate records of vacuolar degenerative changes were shown; **E): Therapeutic:** exhibit moderate focal areas of hepatocellular necrotic, depress infiltrated with mononuclear inflammatory cells mixed with hyperchromatic tumor cells.