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Andrographolide and Resveratrol Effect against Hepatocellular Carcinoma induced in Male Albino Rats.

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) remains one of the most prevalent malignancies worldwide, is a leading cause of cancer- related mortality. Andrographolide (ANDRO) and Resveratrol (RSV) are naturally derived polyphenols that showed promising chemo-preventive effects against HCC. ***Aim:*** This study aims to elucidate the antitumor, antioxidants, anti-inflammatory and anti-apoptotic effects of Andrographolide or Resveratrol single or in combination in induced hepatocellular carcinoma in rats. ***Materials & Methods:*** Swiss male adult albino rats were divided into 6 groups: Group I served as negative control; Group II served as DMSO control ; Group III (HCC group); rats were injected with a single dose of Diethylnitrosamine (200mg/kg.bw) for 5 weeks, Group IV (Andrographolide treated group) rats treated orally with Andrographolide (25 mg/kg.bw) daily for 10 days; Group V (Resveratrol treated group) rats treated orally with resveratrol (2.5 mg/kg.bw) daily for 10 days; Group VI rats treated with combination of Andrographolide and Resveratrol. Blood and tissues samples were collected for some biochemical. ***Results:*** DEN induced HCC that characterized by alterations in liver functions, enhanced the levels of antioxidant in liver tissue, and induced oxidative stress and inflammation. Our data showed that Andrographolide and Resveratrol could ameliorate liver injury (alanine transferase, aspartate transferase, and Galectin 3), up-regulate antioxidant systems (decreasing Malondialdehyde, Nitric oxide and increasing superoxide dismutase, glutathione reduced, glutathione –S-Transferase), and reduce IL-6 level. ***Conclusions:*** Both natural products have high antioxidant and anti-tumor activities against DEN-induced HCC in rats when used alone, while combination blocked to their effects, so we recommended used it in a single state.

Introduction

Liver cancer, particularly Hepatocellular carcinoma (HCC), almost always develops on the background of a chronically diseased liver tissue, induced by long-term exposure to an inflammatory stimulus, such as hepatitis viral infections, excessive alcohol consumption, or metabolic syndrome [(1)], food additives, and water pollutants, environmental and industrial toxic chemicals, as well as several dietary carcinogens, such as aflatoxins and nitrosamines [(2)].

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Diethylnitrosamine (DEN) is a well-known hepatocarcinogenic agent present in tobacco smoke, ground water, cheddar cheese, alcoholic beverages and agriculture chemicals [3]. The rat model of DEN-induced HCC is considered as one of the most accepted and widely used experimental models to study Hepatocarcinogenesis. DEN metabolism in the liver by Cytochrome isoform 2E1 (CYP 2E1) generates reactive oxygen species (ROS) causing oxidative stress; induces chromosomal aberrations, micronuclei and sister chromatid exchanges in the liver. These mutations induced by DEN are responsible for the development of Hepatocarcinogenesis [4].

The conventional therapy of hepatocarcinoma including chemotherapy, radiation, surgical resection and ablation gives little hope for restoration of health because of poor diagnosis and serious side effects; Therefore, developing more effective and less toxic anticancer agents, including natural products, is necessary to prevent or retard the process of Hepatocarcinogenesis [5].

These natural compounds include Andrographolide (ANDRO) and resveratrol (RSV), Andrographolide is a labdane diterpene lactone isolated from the leaves of the *Andrographis paniculata* plant, [6] has the chemical structure 3-[2-[decahydro-6-hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylene-1-napthalenyl]ethylidene]dihydro-4-hydroxy-2(3H)-furanone. ANDRO contains an α-alkylidene γ-butyrolactone moiety and three hydroxyls at C-3, C-19 and C-14 responsible for the cytotoxic activities of Andrographolide against many cancer cell lines [7]. ANDRO has a variety of pharmacological activities, including anti-inflammatory, antidiarrhoeal, antiviral, anti-malarial, cardiovascular, and anticancer and immunostimulatory, hepatocyteprotective activities [8].

Resveratrol (RSV) (3, 5, 4' -trihydroxystilbene) is natural antioxidant polyphenol compound contained in a variety of plants, such as grapes, peanuts, berries and especially in the dried roots of a traditional Chinese medicine *Polygonum cuspidatum* [9]. Its ability to suppress cell proliferation, induce apoptosis and suppress the metastasis and invasion in a number of cell lines has prompted a large interest from people for its use as an anti-tumor component [10].

The present study investigate the antitumor, antioxidant, anti-inflammatory and anti-apoptotic effects of Andrographolide or Resveratrol single or in combination in induced Hepatocellular carcinoma in rats. Also, study the side effects of these natural compounds on biochemical alternation associated with HCC.

Materials & Methods

48 adult male Swiss albino rats weighing (80-100 g) were housed at experimental animal house of the Faculty of Science, Zagazig University. The animals were maintained in controlled environment of temperature, humidity, light, and fed on a commercial standard diet and tap water *ad libitum*.

Chemicals

Diethylnitrosamine (DEN), Andrographolide, Resveratrol were purchased from Sigma-Aldrich Chemical Co., (St Louis, MO, USA), Galectin 3 (Gal-3) a sandwich ELISA Kit method from (BG Medicine, Waltham, MA**)**.

Induction of Hepatocellular carcinoma (HCC)

Diethylnitrosamine was freshly dissolved in sterile saline and intrapretonially (I.P.) injected into rats at a dose 200 mg/kg b.w. for 5 weeks [11].

Experimental design

48 adult male Swiss albino rats were divided into 6 groups (8 rats/each) as follow: *Group I: Negative Control (N.C.)*: served as normal control group injected with sterile saline for 5 weeks, *Group II(DMSO group*): rats were injected I.P. with (0.2%)DMSO for 10 days [12] , *Group III(HCC group):* rats were injected I.P with single dose of DEN (200 mg/kg.bw) for 5 weeks, *Group IV(Andrographolide treated group) (DEN+ Andrographolide)*: rats were administrated with Andrographolide orally at a dose (25 mg/kg.bw) daily for 10 days [13], *Group V (Resveratrol treated Group) (DEN+ Resveratrol)*: rats were administrated with Resveratrol at a dose (2.5mg/kg.bw) daily for 10 days [14], and *Group VI(Combination treated Group) (DEN+ Andrographolide + Resveratrol)*: rats were orally administrated with a combination dose of Andrographolide and Resveratrol daily for 10 days. At the end of the experiment, animals were weighed then anaesthetized under light di-ether

and dissected. Blood samples and liver tissues were collected for biochemical analysis.

Biochemical analysis

Anti-oxidant assays:

The plasma samples were collected for different antioxidant assays. Superoxide dismutase (SOD), glutathione –S- transferase (GST), reduced glutathione (GSH), and malondialdhyde (MDA), Nitric Oxide (NO) levels were determined by using Bio-diagnostic kit method according to the methods of **Nishikimi *et al.*,** [(15)], **Habig *et al.*,** [(16)], **Beutler *et al.*,** [(17)], **Satoh,** [(18)] and **Montgomery & Dymock** [(19)] ; respectively.

The activities of aspartate transaminase (AST) and alanine transaminase (ALT) were estimated by the method of **Karmen *et al.*,** [(20)].

***Gal-3 and Interleukin-6 measurement*:** Galectin-3 (GAL-3) level was determined by using a sandwich ELISA Kit method described by **Christenson *et al.*,** [(21)].

IL-6 antigen was determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit from Promocell (Heidelberg, Germany) according to the method of **Isomura *et al*.**, [(22)].

Statistical analyses:

Data were evaluated by one-way analysis of variance (ANOVA)by "SPSS" 14.0 for Microsoft Windows, SPSS Inc. [(23)] and considered statistically significant at a two-sided P < 0.05.Numerical data were expressed as mean ± SD.

Results

Effect of Andrographolide and resveratrol on antioxidants in all studied groups:

The mean value of MDA and NO levels were found to be 16.75±1.26 (nmol/ml), and 24.34±3.35 (µmol/ l) in negative control group; respectively. HCC group showed a significant increase in both MDA levels to be 42.36 ±1.94 (nmol/ml) by 886.25%, and NO levels to be 60.39 ±4.33 (µmol/l) by 148.13%, ($p<0.001$) compared to negative control group. While, administration of andrographolide, resveratrol alone or in combination resulted in a significant decrease in MDA levels to 5.87 ±0.07, 6.61±0.06, and 16.21±0.33 (nmol/ml) by 86.15%, 84.41%, and 61.74%, ($p<0.001$) respectively; compared to the HCC group. Also, NO levels were decreased significantly in Andrographolide, resveratrol and combination groups to 9.41 ±0.71, 12.06±1.32, and 22.44±2.01 (µmol/l) by 84.41%, 80.03%, and 62.84%, respectively, ($p<0.001$) compared to HCC group, fig (1, 2).

On the other hand; SOD, GSH and GST activities were decreased from 293.08±3.03 (U/ml), 9.08±0.67 (nmol/ml), 267.20±21.05 (U/l) in negative control group to 99.11 ±5.08 , 4.16±0.49 , 126.39±16.19 in HCC group by 66.18%, 54.15% and 52.69% ;respectively, ($p<0.001$). While, their activities were significantly increased to 745.76±66.44, 17.75±0.85 and 710.56±57.70 in Andrographolide group, to 599.84 ±45.39, 13.14±1.12 and 512.59±7.99 in resveratrol group, and to 329.45±1.86, 8.72±0.09, and 385.43±5.33 by 232.40%, 109.55%, and 204.95% in combination group; respectively, ($p<0.001$) compared to HCC group, fig (3, 4, 5).

Effect of Andrographolide and resveratrol on liver enzymes in all studied groups:

Measurement of liver enzyme activities demonstrated significant increase in ALT, and AST activities in HCC group to 114.45 ±1.94 , and 176.45 ±12.52 (U/L) by 105.48% and 48.90%,; respectively compared to negative control group, ($p<0.001$). These high activities of liver enzymes were significantly reduced to 23.30 ±4.14, and 93.80 ±1.15 by 79.64%, and 46.84%; respectively in andrographolide group, to 37.06±0.08 and, 106.44±1.12 by 67.62%, and 39.68% in resveratrol group, and to 50.9375±0.63682, and 142.625±2.9016 by 55.49%, and 19.17% in combination group; respectively, ($p<0.001$) compared to HCC group, fig (6, 7)

Effect of Androgapholide and resveratrol on Gal-3 concentrations and Interleukin- 6 level in all studied groups:

Gal-3 concentration was significantly elevated in HCC group to 22.16 ±1.74 (ng/ml) compared to negative control group 3.76±0.08 (ng/ml) by 490.11% ($p<0.001$), fig (4). Meanwhile, Gal-3 was significantly decreased to 2.56±0.62 by 88.84% in andrographolide group, to 3.74±0.54 (ng/ml) by 83.12% in resveratrol group, and to 7.0±0.79462 (ng/ml)

by 68.41% in combination group; respectively, ($p<0.001$) compared to HCC group, fig (8).

In HCC group there was highly significant ($P < 0.001$) increase in serum interleukin-6 by 10.58±1.22 (pg/ml) by 233.07 % as percent change from normal control group. HCC groups treated with andrographolide, resveratrol or their mixture showed highly significant ($P <0.001$) decrease in IL-6 level by 0.98±0.15, 1.97±0.21, and 4.06±0.47 (pg/ml) by 90.74%, 81.41%, and 61.62%; respectively as compared with HCC control group. fig (9).

Correlations between different Studied Parameters among studied groups:

There were significant positive correlations between Gal-3 & IL-6, ALT; while, there were significant negative correlations between Gal-3 & SOD, and also, significant negative correlations were found between IL-6 &GST (Fig 10).

Discussion

Natural compounds, particularly those obtained from plants, are plant polyphenols derivatives that have been characterized in several cell culture and animal cancer models with antitumor effects [(24)].

Andrographolide is used extensively as the traditional Chinese medicine [(25)] where it possesses anticancer, antioxidant and hepato-protective activities, also exhibits many biological activities such as antibacterial , anti-inflammatory, anti-malarial , immune-modulation , antithrombotic , and anti-hepatitis activity [(26)]. Also Resveratrol, a polyphenol derived from the roots of *polygonum cuspidatum* Sieb. Et Zucc, has a number of biological activities as it possesses cancer-chemopreventive and cytostatic properties via the three major stages of carcinogenesis, i.e. initiation, promotion and progression [(27)].

DMSO has been known as an organic solvent for lipophilic drugs. It plays multiple roles on cellular functions (e.g., metabolism and enzymatic activity) and cell growth by affecting cell cycle and apoptosis [(28)]. According to our present data, we found that DMSO is safe without side effects on liver tissues when compared to negative control.

In this study, we found that Andrographolide increase hepatic antioxidant activity; these results indicate that Andrographolide enhances antioxidation capacity in rat liver. This in line with Chen *et al*., [(29)] who proved that hepatic glutathione (GSH) content, superoxide dismutase (SOD), and GST activities increased by andrographolide protection against CCL_4 – induced liver damage. Also, Resveratrol was found to be a very potent anti-oxidant [(30)]. Based on various experimental and theoretical results it is definitely concluded that the phenolic (-OH) plays a major role in the activity of resveratrol [(30)]. Also, Resveratrol has also been shown to quench reactive oxygen species and scavenge superoxide anion radicals and hydroxyl radicals and strongly inhibits nitric oxide (NO) production by down-regulating inducible nitric oxide synthase gene expression; Due to its lipophilic character, resveratrol is able to bind the lipoprotein particles suggesting that this vent improved its antioxidant activity [(30)]. Resveratrol prevents lipid peroxidation by chelating copper and by scavenging ROS. The efficiency and action mechanism of trans-resveratrol is due to the para-hydoxyl groups shows a greater radical-scavenging activity than the meta-hydroxyl groups of trans-resveratrol [(31)]. It has been reported that due to hydoxylated structure of resveratrol, it can form a radical derivative stabilized by the delocalization of two electrons between the two aromatic cycles and the methylene bridge joining these two cycles [(32)].

Our results are in agreement with many authors; Study by Ates *et al*., [(33)] confirms that an elevation in GSH level is due to the free radical scavenging properties of resveratrol [(30)] demonstrated that the presence of resveratrol showed the decrease in the MDA level and the protein carbonyl group content. Study by Kirimlioglu *et al*., [(34)] who reported that MDA levels in liver tissue and plasma were higher in group subjected to 70% partial hepatectomy than those of group treated with resveratrol before and after 70% partial hepatectomy. Cetin *et al*., [(35)] who demonstrated that an increased level of malondialdehyde (MDA) in

Methotrexate- induced oxidative liver injury has been reversed by resveratrol administration.

Our results in a harmony with many authors; Youn *et al.*, [36]; Zong *et al.*, [37] who reported that resveratrol is able to inhibited IFN-γ and iNOS protein expression and down-regulate NO production induced by various inflammations. Also, Chiou *et al.*, [38] who reported that andrographolide showed a significant reduction in NO production.

Also, Resveratrol was found to be a highly potent antioxidant that could inhibit free radical generation in kidney, liver. Elevation in glutathione level may be due to the free-radical scavenging properties of resveratrol [33]. Our results are consistent with Kirimlioglu *et al.*, [34] study who reported that there are Significant increases in tissue levels of reduced glutathione (GSH) levels was observed.

In this study, Andrographolide increase hepatic GSH levels and SOD activity. These results indicate that Andrographolide enhances antioxidation capacity in rat liver. This in line with Chen *et al.*, [29] which proved that GSH and SOD activity increased by andrographolide protection against CCL_4 – induced liver damage. This result is consistent with previous reports that indicated Andrographolide showed free radical scavenging properties and protective effects on hepatotoxicity induced in mice by CCl_4. Also, the treatment with resveratrol showed a significantly reduction in glutathione level; as elevation in glutathione level may be due to the free-radical scavenging properties of resveratrol according to Ates *et al.*, [33].

Serum liver biomarkers (ALT, AST) are important criteria for the evaluation of liver toxicity. The amounts of enzymes that leak into the blood stream indicate the severity of hepatic damage [39]. The increased serum levels of AST and ALT are due to the damage to the structural integrity of the liver, since these enzymes are normally located in the cytoplasm and released into the circulation after cellular injury [40].

In the present study, it was observed that, rats treated with DEN showed elevated serum markers such as ALT, AST activities. There is an agreement between the data obtained in this study and that obtained by Ko and Lim**,** [41] who detected a marked elevation in aminotransferases enzymes (ALT and AST) in the serum of the CCL_4 intoxicated rats.

These results were in a harmony with many studies; Nasir *et al.*, [42] who reported that the treatment with Andrographolide show reduction in the levels of ALT and AST towards the normal value is an indication of regeneration process. Chen *et al.*, [29] reported that Andrographolide decrease activity of ALT and AST. Also, our study revealed that resveratrol treatment significantly attenuated the increased activities of these enzymes compared to HCC group; demonstrating the protective effect of this polyphenol against the induced liver damage. These results are in agreement with those found in studies using resveratrol by Atmaca *et al.*, [43] who examined the hepato-protective effect of resveratrol, decreasing AST and ALT enzyme activity which can be attributed to the capability of resveratrol to conserve the membrane integrity of cellular organelles.

Galectin-3 (Gal-3) is a member of an evolutionarily conserved family of soluble β-galactosidee binding lectins that play a key role in several diverse biologic processes and disease states Fiuzat *et al.*, [44]. Gal-3has been found to be involved in many biological processes, such as cell-cell and cell-extracellular matrix adhesion, cell growth and differentiation, the cell cycle, signaling, apoptosis and angiogenesis [45]. According to our present data, we can confirm that both natural products (andrographolide and resveratrol) have anti-tumor properties.

Interleukin-6 (IL-6), a multi-functional cytokine, the level of IL-6 and its receptor expression has been consistently related to the progressing stages of cancer and is most significant at benign hyperplasia and metastasis, and up-regulation level of IL-6 has been observed in HCC, which suggested that IL-6 might be related with the risk of HCC. Furthermore with the increasing tumor mass the IL-6 levels become significantly high and

thus can be targeted for anti-cancerous drug designing [46].

Also, these results were in a harmony with many studies; Dias *et al*., [47] studied the effect of Resveratrol and found that it caused a decrease in interleukin 6 (IL6) in mouse serum. Results reported by Zheng *et al*., [46] showed that resveratrol down-regulated the expression of inducible nitric oxide synthase (iNOS) and interleukin-6 (IL-6), therefore, suppressed the production of nitric oxide and the secretion of IL-6 in LPS-stimulated RAW264.7 cells.

Conclusion:

Although combined administration of ANDRO and RSV significantly exert a potential chemo-preventive effect, but individual administration was more effective in preventing HCC development. These novel findings suggest that both natural compounds have an antagonistic effect suggesting concerted efforts are needed to identify the most optimal combinatorial strategies.

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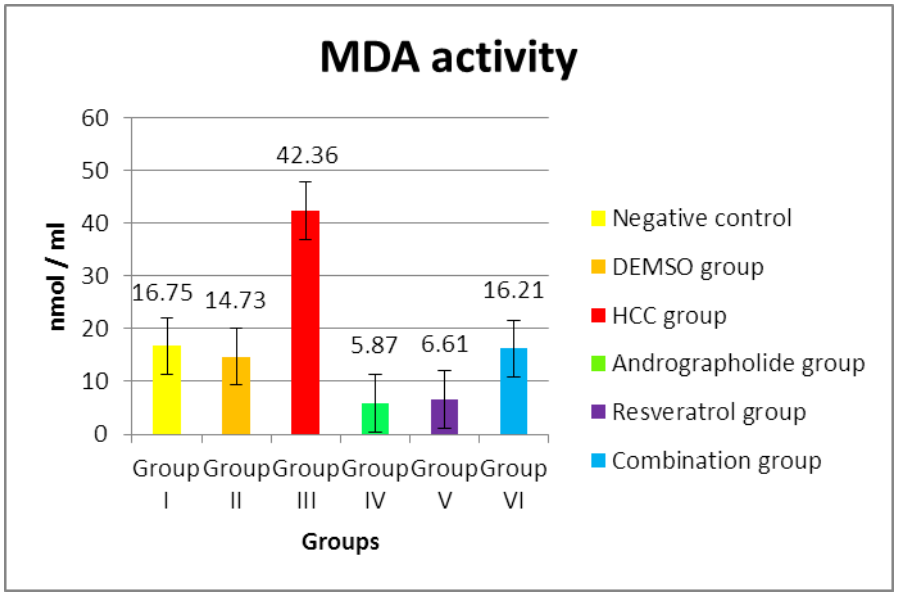

Fig. (1): Effect of andrographolide and resveratrol on MDA activities in all studied groups

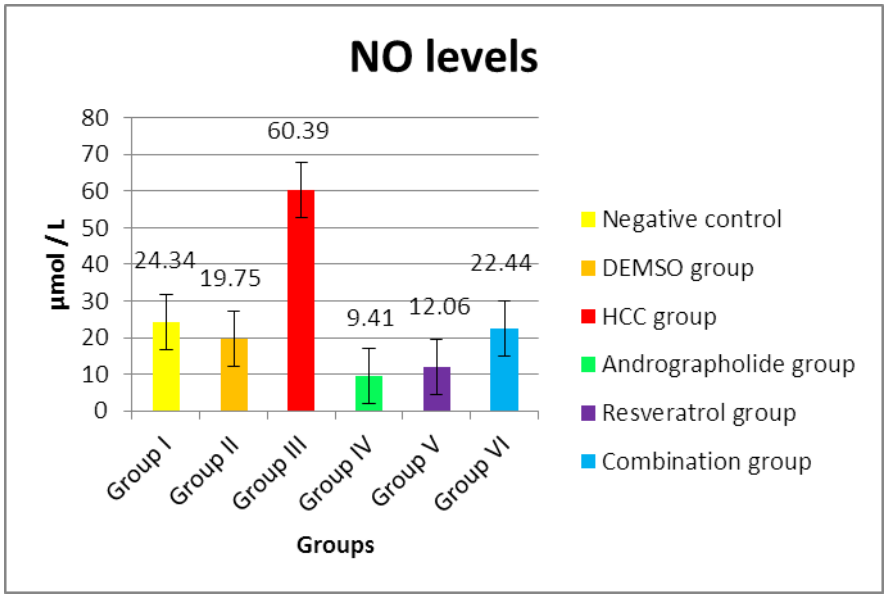

Fig. (2): Effect of andrographolide and resveratrol on NO levels in all studied groups

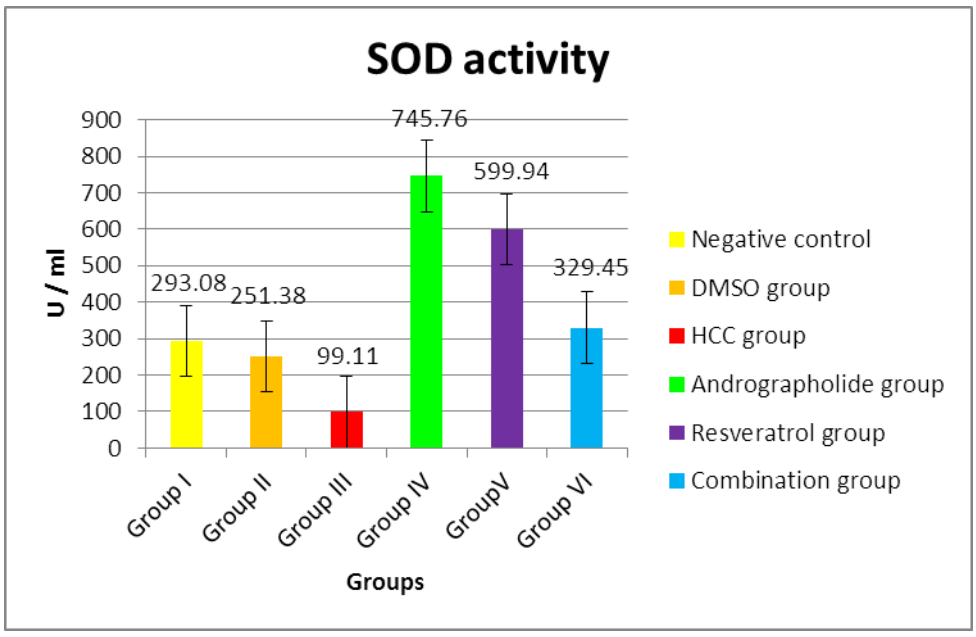

Fig. (3): Effect of andrographolide and resveratrol on SOD activity in all studied groups.

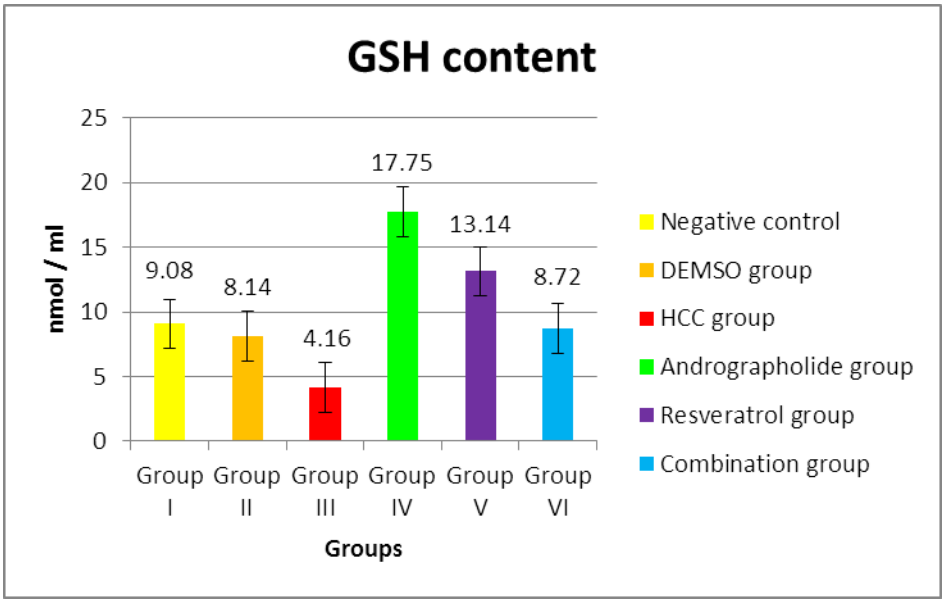

Fig. (4): Effect of andrographolide and resveratrol on GSH content in all studied groups.

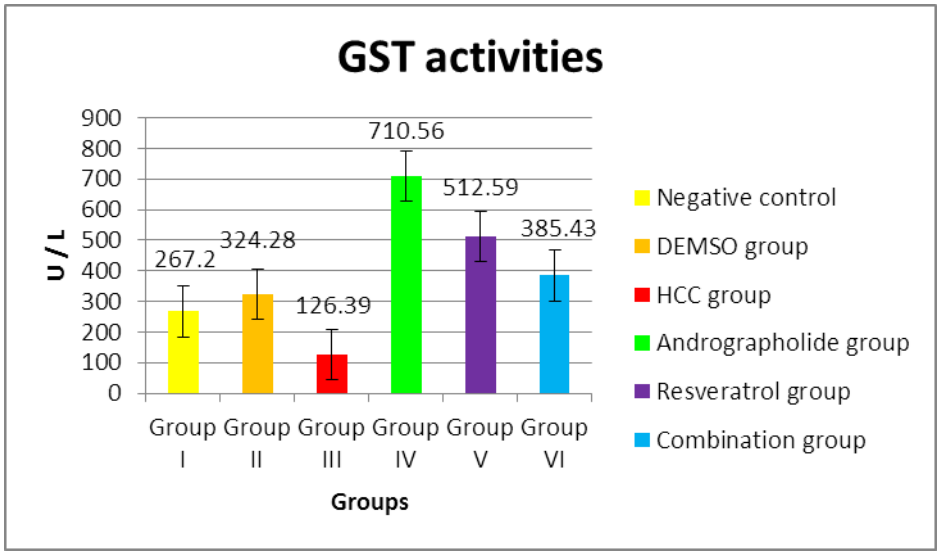

Fig. (5): Effect of andrographolide and resveratrol on GST activities in all studied groups.

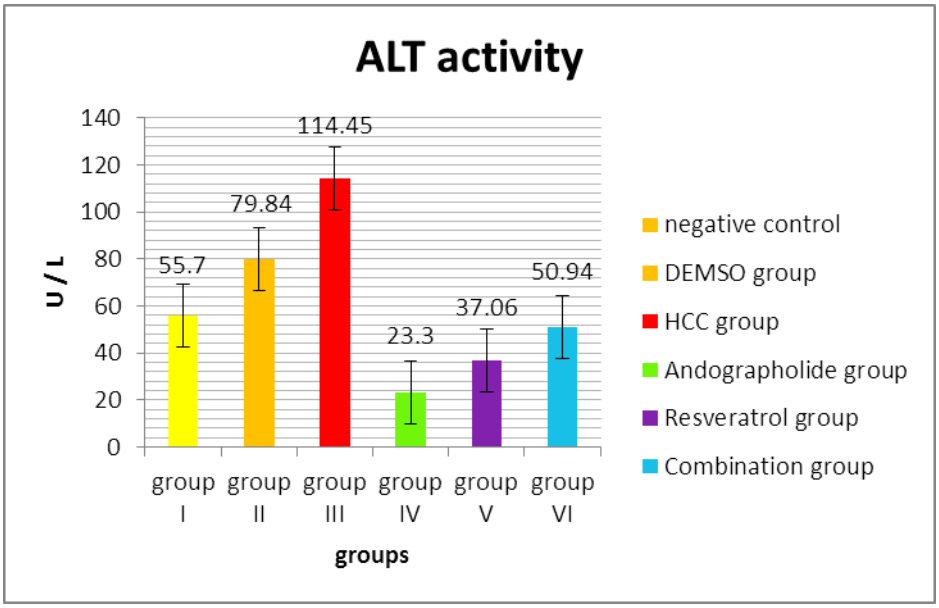

Fig. (6): Effect of andrographolide and resveratrol on ALT activity

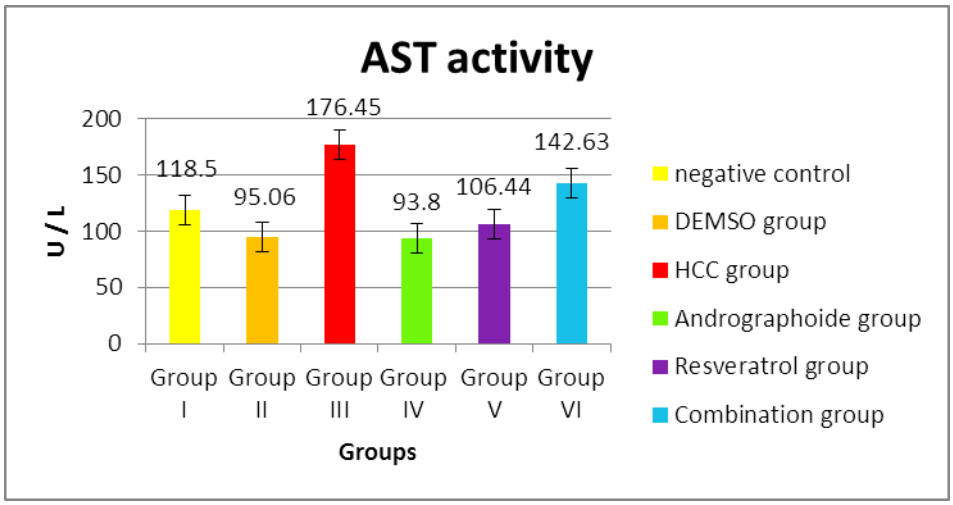

Fig. (7): Effect of andrographolide and resveratrol on AST activity

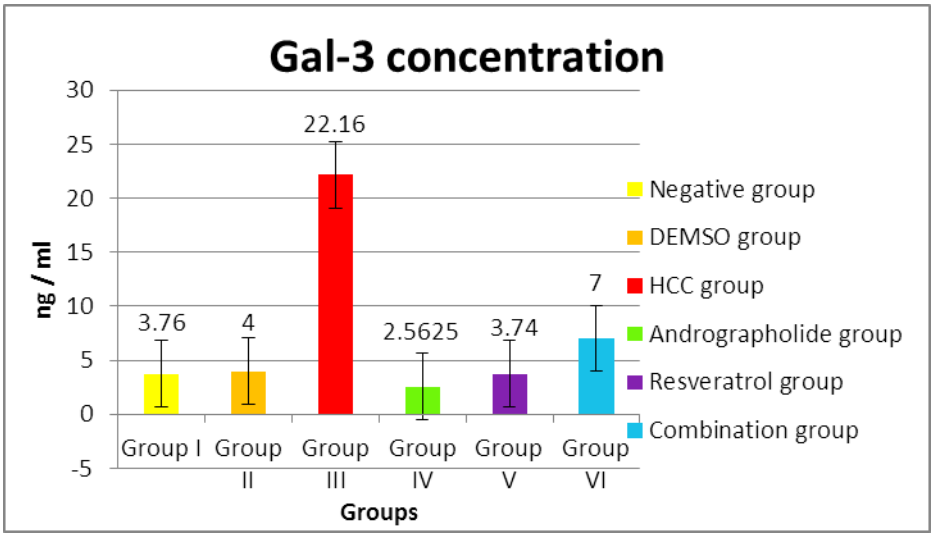

Fig. (8): Effect of andrographolide and resveratrol on Gal-3 concentrations in all studied groups.

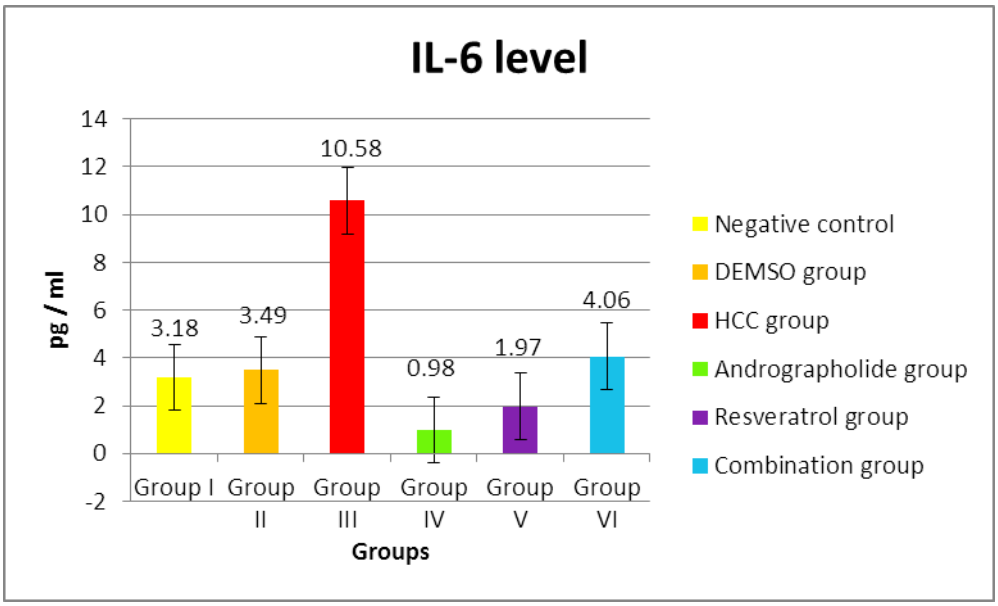

Fig. (9): Effect of andrographolide and resveratrol on IL-6 levels in all studied groups.

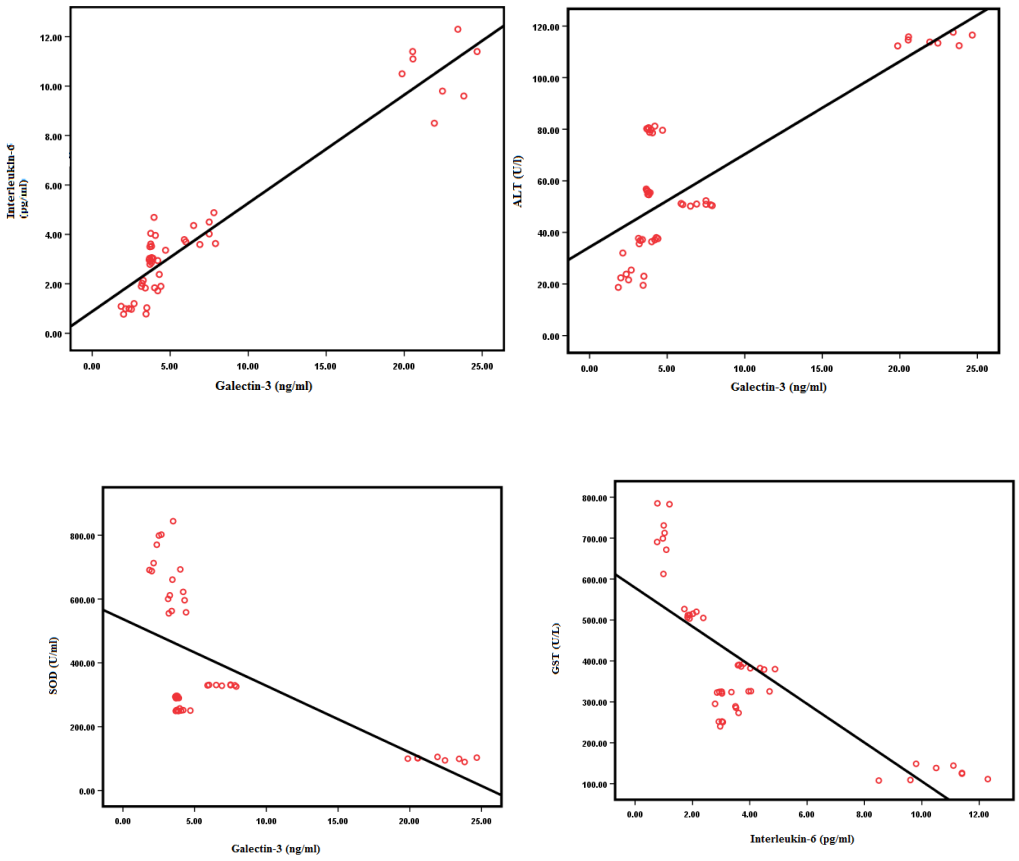

Fig. (10): Correlations between different Studied Parameters among studied groups