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EXAMINING THE HEPATOPROTECTIVE EFFECTS OF GRAPE SEED EXTRACT AND THE ANTI-ESTROGENIC POTENTIAL OF LETROZOLE: EXPRESSING AROMATASE GENE PATTERN IN N-ETHYL-NITROSAMINE-INDUCED HEPATOCELLULAR CARCINOMA USING AN ANIMAL MODEL.

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Abstract
The study investigates the inhibitory effects of grape seed extract on HepG2 cell proliferation, focusing on processes like autophagy and the upregulation of proteins linked to the mitogen-activated protein kinase (MAPK) pathway. Extending previous research, it examines the effects of grape seed extract alone and in combination with letrozole on N-ethylnitrosamine-induced hepatocellular carcinoma in male albino Wistar rats. Employing a quasi-experimental design with purposive sampling, fifty rats were divided into five groups. Significant variations ($p < 0.001$) in serum albumin and liver enzyme levels were observed among groups through one-way ANOVA analysis. Additionally, a notable disparity ($p < 0.05$) was found in the relative gene expression of the aromatase gene. The results indicate significant impacts of both letrozole and grape seed extract on liver enzyme levels and highlight Group C as showing restored gene expression ($p < 0.05$). This study underscores the potential therapeutic effects of grape seed extract and letrozole in mitigating hepatocellular carcinoma progression, emphasizing the importance of further investigation into their mechanisms and clinical applications.

Keywords: Mitogen-Activated Protein Kinases, Grape Seed Extract, Hepatocellular Carcinoma, Hepatocyte, carcinoma, aromatase

Introduction

Cancer ranks as the second leading cause of death globally, with approximately 18.1 million new cases and 9.6 million deaths reported in 2018[1]. Universally, hepatocellular carcinoma (HCC) stands as the fifth most common form of primary liver cancer. The prevalence of HCC in Sri Lanka is escalating, with direct associations observed with conditions such as cirrhosis, hepatitis B, and hepatitis C infections[2]. The documented occurrence rate of hepatocellular carcinoma (HCC) in

Pakistan stands at 7.6% per 100,000 males and 2.8% for females. Significantly, chronic hepatitis C virus (HCV) infection accounts for 60-70% of all HCC instances in Pakistan, distinguishing it from neighboring Asian countries where chronic hepatitis B virus (HBV) infection is more prevalent[3]. The notable increase in hepatocellular carcinoma (HCC) occurrence underscores the imperative necessity for targeted initiatives aimed at mitigating the disease burden and elucidating efficacious intervention methodologies[4]. The elevated mortality rate associated with hepatocellular carcinoma (HCC) primarily stems from a bleak prognosis and the inadequate accessibility of efficacious therapeutic modalities. Commonly utilized treatment approaches for HCC encompass radiation therapy, chemotherapy, and surgical resection[5]. Chemotherapy shows promise as a viable treatment for individuals with HCC, either on its own or alongside other therapies, despite the somewhat limited effectiveness of the current options available[6]. Moreover, radiation therapy holds significant importance as it endeavors to diminish the tumor's dimensions before surgical excision or eradicate residual cancer cells post-surgery, thereby enhancing the overall efficacy of treatment regimens[7]. Traditional oncologic interventions are extensively employed globally, yielding considerable enhancements in survival rates for numerous patients. Nevertheless, certain individuals experience only partial remission alongside an array of adverse symptoms. Furthermore, the exorbitant expenses associated with many treatment protocols render them financially inaccessible to certain populations, particularly those residing in underdeveloped regions[8]. For such fatal liver cancer, the patient requires new treatment choices. The use of natural combinations may provide patients with improved outcomes with lesser systemic toxicity and fewer side effects[9]. On a global scale, grapes are extensively ingested fruits due to their abundant polyphenol content, which confers a bolstering effect on the body's innate health. Grape seed extract proffers advantages counteracting various ailments such as inflammation, cardiovascular maladies, hypertension, diabetes, cancer, peptic ulcers, microbial infections etc[10]. The effects of grape seed involve the induction of apoptosis, suppression of cellular proliferation, mitigation of oxidative stress, and modulation of inflammatory markers through downregulation[11]. Moreover, grape seed proanthocyanidins (GSPs) have also exhibited inhibitory effects on phosphatase activity during liver regeneration[12]. Earlier investigations have highlighted a robust correlation between the anti-proliferative attributes of grape seed proanthocyanidins (GSPs) on HepG2 cells and processes such as autophagy[13]. Similarly, there has been observation regarding the enhancement in the expression of proteins linked to the mitogen-activated protein kinase (MAPK) pathway[14].

The CYP19A1 gene encodes the enzyme aromatase, thus facilitating the conversion of androgens to estrogens. Aromatase is implicated in diverse biological functions including proliferation, metabolism, and hormone signaling. Additionally, it is upregulated in several cancers, leading to the growth of hormone-dependent tumors[15]. The Food and Drug Administration (FDA) has sanctioned letrozole (marketed as Femara), an aromatase inhibitor, as a primary pharmacological intervention for aromatase-positive cancers[16]. Aromatase inhibitors exert their anti-estrogenic effects by impeding cytochrome P450 activity[17]. This modulation are accomplished through reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemical techniques[18]. Consequently, by viewing prior research, the current investigation aims to assess the impact of grape seed extract administration alone or combined with letrozole on hepatocellular carcinoma. This assessment will involve analyzing albumin levels, liver enzyme activity, and the expression of the aromatase gene in albino Wistar rats induced with hepatocellular carcinoma. If this is proven then it may be advised in routine prescription to improve the hepatocellular carcinoma prognosis by a simple, harmless, and cost-effective method. The study will also raise awareness to the doctors and society to go for regular follow-ups whose parents and siblings developed hepatocellular carcinoma, for their early detection of the aromatase gene.

Methodology

Study Design:

Quasi-experimental.

Study Location:

The research was conducted at the Diagnostic & Research Laboratory of Isra University Hyderabad and the Animal Husbandry and Veterinary Sciences Department of Sindh Agriculture University Tando Jam.

Study sampling and eligibility criteria

The study's criteria for inclusion and exclusion employed non-probability (purposive) sampling to select 50 fully developed male albino Wistar rats for the sample pool. Inclusion criteria encompassed male albino Wistar rats of mature age weighing between 200 and 250 grams, and exhibiting good health. Rats falling outside the specified weight range, female rats, those with inadequate dietary intake, and rats displaying signs of illness or impending death were all excluded from the study.

Experimental Details

A total of n=50 rats were divided into five groups n=10 rats in each group. The details were as follows:

- Group A (control): Received only distilled water.
- Group B (control-experimental): Received intraperitoneal injections of N-ethylnitrosamine (30mg/kg bodyweight) diluted in 0.9% normal saline twice a week for 11 weeks[19].
- Group C (experimental): Received intraperitoneal injections of N-ethylnitrosamine (30mg/kg bodyweight) in 0.9% normal saline twice a week and oral grape seed extract (100mg/kg) daily for 11 weeks[20].
- Group D (experimental): Received intraperitoneal injections of N-ethylnitrosamine (30mg/kg bodyweight) in 0.9% normal saline twice a week and oral letrozole (2mg/kg body weight) daily for 11 weeks[21].
- Group E (experimental): Received intraperitoneal injections of N-ethylnitrosamine (30mg/kg bodyweight) twice a week, oral letrozole (2mg/kg body weight) daily, and oral grape seed extract (100mg/kg) daily for 11 weeks.

After an additional week of acclimatization, all animals underwent anesthesia via subcutaneous administration of Ketamine (20mg/kg) and Xylazine (2mg/kg) to alleviate discomfort. Blood samples were then collected via cardiac puncture using EDTA and Plain tubes, with the sera extracted for biochemical analysis, specifically liver function testing. Subsequently, all animals were euthanized through cervical dislocation, followed by laparotomy. During laparotomy, the weight of each rat's liver was recorded, and the livers were preserved in 10% formaldehyde. Liver tissue samples were procured for Reverse Transcriptase PCR analysis.

Outcome Measures

Liver Enzyme levels

Conventional biochemical tests were employed to assess the levels of Albumin, ALT, AST, and GGT. into the sample tray of Hitachi Rosche, cobas 311 automatic analyzer.

Aromatase Gene Expression

A meticulous three-step process was employed to extract the expression of the Aromatase gene from rat liver tissue. Initially, total RNA was purified from male albino rat liver tissues using the Gene JETTM RNA Purification Kit (Thermo Scientific) following the manufacturer's guidelines[22-23]. The liver tissues underwent careful preparation, homogenization, proteinase K treatment, and multiple washes to isolate the RNA. Subsequently, the purified RNA was utilized for cDNA synthesis using

the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific)[24]. The final step involved amplifying the Aromatase gene via PCR post-cDNA synthesis. For efficient gene amplification, the Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2X) was employed, along with specifically designed primers for the Aromatase gene as under[25],

Primer	Forward	Reverse
RAT GAPDH	**ATg ACT CTA CCC Acg gCA Ag**	**AGT TGT CAT GGA TGA CCT TGG**
RAT GAPDH	**ATg ACT CTA CCC Acg gCA Ag**	**ggA AgA Tgg TgA Tgg gTT TC**
RAT AROMATASE	**Agg ACA CAC ATg CTC ACA CAC**	**TgT AgA Agg gTA CAg TCT GGG**

The cDNA template was incorporated into the master mix reaction, which underwent a thermal cycling program in a PCR cycler. This process involved denaturation, annealing, and extension steps on the rat liver tissue to amplify the Aromatase gene[26].

Results

Table 1. A total number of n=50 male albino Wistar rats were included in this study. The rats were equally allocated into one of five groups. To determine the effects of grape seed extract and letrozole on levels of albumin and liver enzyme among N-diethylnitrosamine-induced male albino Wistar rats' one-way analysis of variance was applied at 95% of the Confidence interval between group analysis and the results revealed that a significant difference in mean $p<0.001$ was found in the levels of albumin between five groups. Similarly, on liver enzyme levels a significant difference between group $p<0.001$ was found in the levels of serum aspartate aminotransferase (AST), serum alanine aminotransferase, and gamma-glutamyl transferase (GT). The analyses of the findings are illustrated in Table 1.

Table 1. Analyses to Determine the Effects of Grape Seed Extract and Letrozole on Serum Albumin and Liver Enzyme Levels of N-eithylnitrosamine induced male albino wistar rats

Variables	Sum of Square SS	Mean sum of Square MS	df	F value	Level of Significance
Albumin	31.68	7.92	4	64.92	<0.001
AST	10271.5	2567.8		217.01	
ALT	1188.2	2970.53		32.65	
GT	26026.5	6506.6		217.52	

Subsequent analysis revealed significant disparities in the concentrations of albumin and liver enzymes among the groups. Notably, by week 11, Group B exhibited markedly lower albumin levels at 1.56 ± 0.29 g/dl compared to the control group (Group A), which registered levels of 3.76 ± 0.26 g/dl. Similar trends were observed in Groups C, D, and E, indicating an impact of the treatments on albumin levels. Furthermore, divergent patterns were observed in the levels of liver enzymes, including ALT, AST, and GT. For instance, at week 11, Group A displayed ALT levels of 27.8 ± 10.22 IU/L, while Groups B, C, D, and E exhibited higher values. Comparable trends were noted for AST and GT levels, underscoring the varied effects of letrozole and grape seed extract on the liver function parameters of the experimental rats as shown in graphs 1,2,3 & 4 respectively.

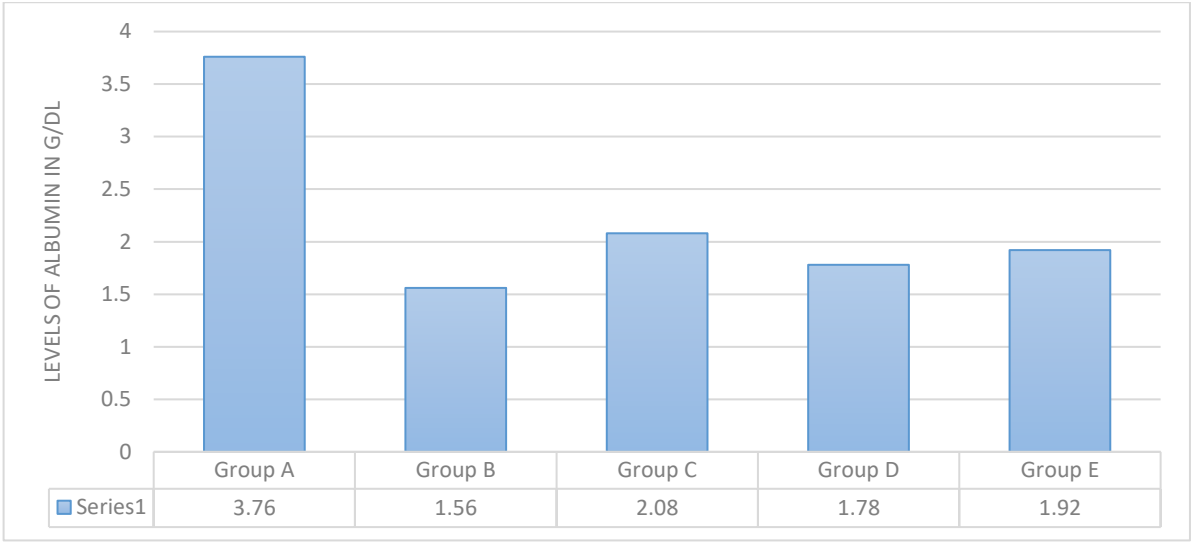
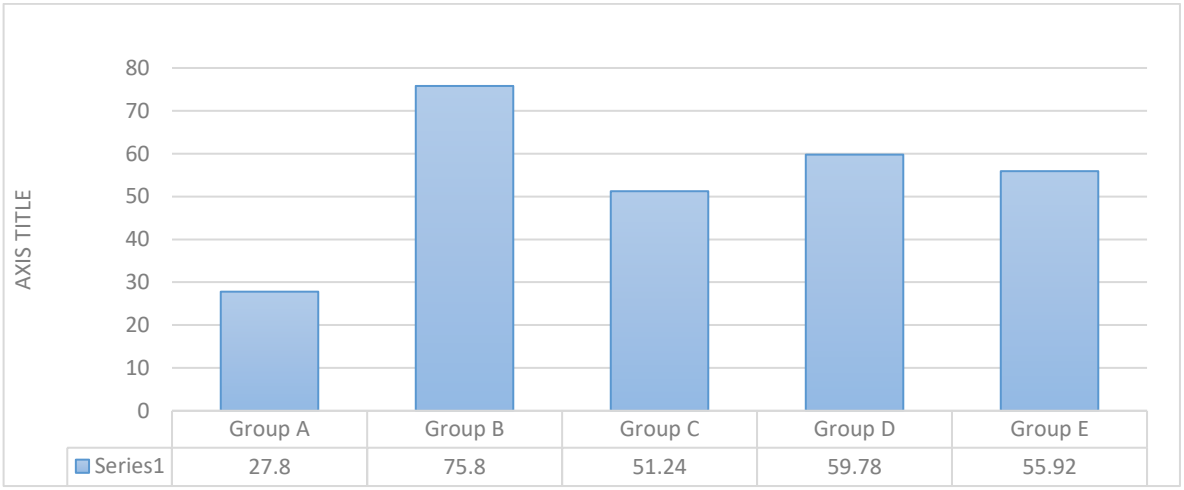


Figure 1. Analyses of the Effects of Grape Seed Extract and Letrozole on Levels of Albumin in g/dl



Analyses of the Effects of Grape Seed Extract and Letrozole on Levels of Alanine Transaminase (ALT) IU/L

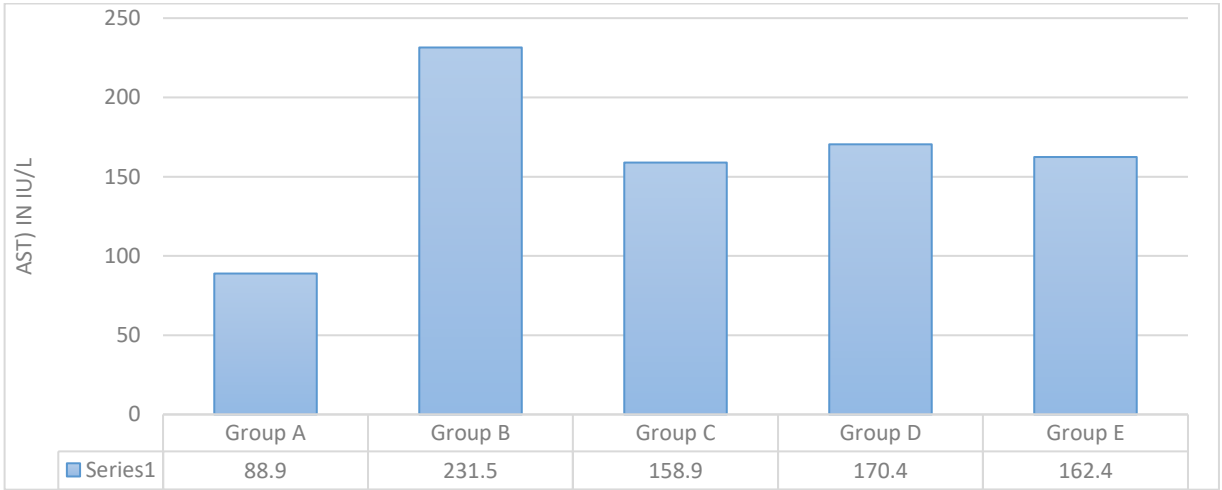


Figure 3. Analyses of the Effects of Grape Seed Extract and Letrozole on Levels of Aspartate Transaminase (AST) IU/L

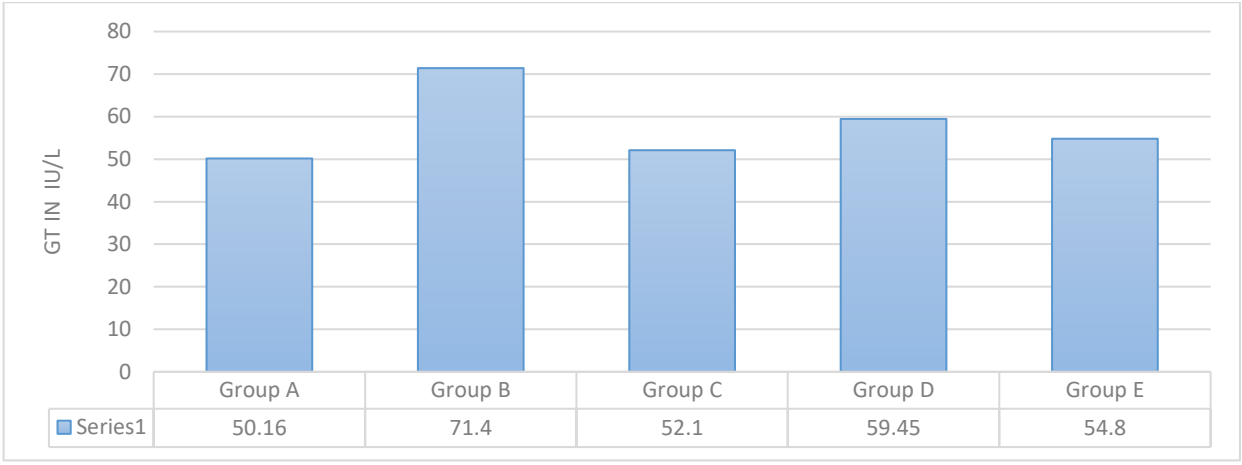


Figure 4. Analyses of the Effects of Grape Seed Extract and Letrozole on Levels of Gama Glutamyl Transferase (GT) IU/L

Table 2. The examination of aromatase gene expression unveiled a notable disparity in mean values among the groups, with a significance level of $p<0.05$. The results indicated that the relative gene expression of aromatase varied across the groups. Specifically, in Group A, the mean expression was 21.13±3.59, while it was markedly reduced to 0±0 in Group B not showing genetic expression. Conversely, Group C displays the restored gene expression. of 28.61±2.71, with even higher values noted in Groups D (36.22±3.15) and E (35.24±3.48).

Table 2. Between-group analyses for determining the effects of grape seed extract and letrozole on relative gene expression

Variables	Relative gene expression	Standard Deviation	df	F value	Level of Significance
Group A	**21.13**	**3.59**			
Group B	**0.0**	**0.0**			
Group C	**28.61**	**2.71**			
Group D	**36.22**	**3.15**	**4**	**46.97**	**<0.001**
Group E	**35.24**	**3.48**			

Expression of the aromatase gene expression pattern in Groups A, B, C, D & E is shown in Figure 5.

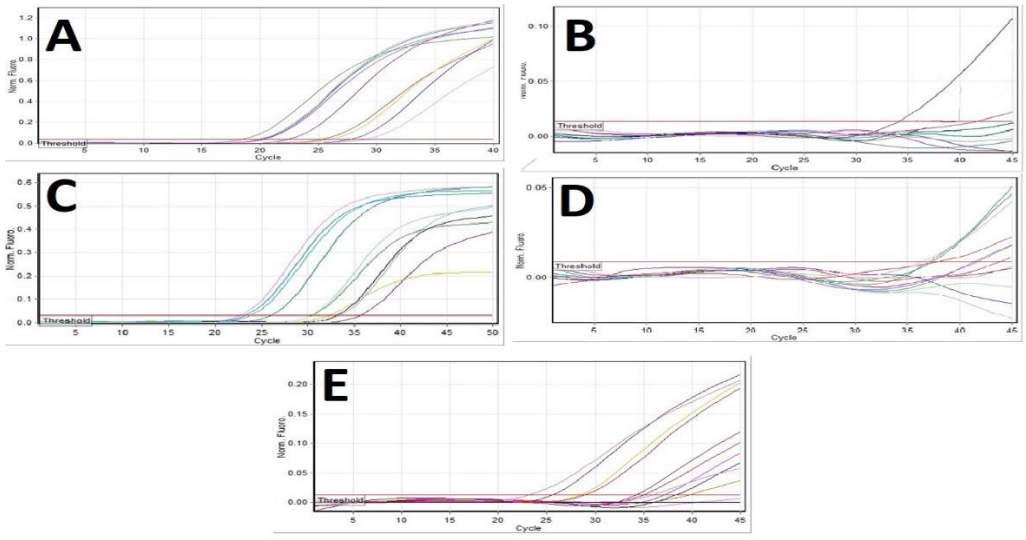


Figure 5. Expression of aromatase gene in experimental groups.

Relative aromatase gene expression in group A has noted as 21.13 in contrast to the experimental groups. B-0, C-28.61, D-36.22 and E-35.24. In group B, aromatase gene did not showed gene expression. Group C was given grape seed extract as treatment showed the up regulation of aromatase gene. Group D, showed relative aromatase gene expression as 36.22 in experimental animals. Letrozole in most of animals showed down regulate of aromatase gene. Relative gene expression in group E was noted as 35.24 combination of GSE letrozole up regulated the aromatase gene.

DISCUSSION

We induced hepatic carcinoma with N-eithylnitrosamine at the dose of 30mg/kg bodyweight twice a week for 11 weeks in the rat model. Ghufran H, et al, 2021 also used rats model for study, injected with N-eithylnitrosamine (DEN) at a dose of 30 mg/kg twice a week for 10 weeks then once a week from 12th to 16th weeks, to explore time-dependent regulatory changes during disease advancement and HCC development. DEN-intoxicated rats manifested inflammation at week 4th, fibrosis at week 8th and cirrhosis with early HCC tumors at week 12th. Molecular analysis revealed that key markers of inflammation, fibrosis, angiogenesis, oxidative stress and pro-apoptotic markers showed significant upregulation[27]. Khan F, et al, 2017; Anti-cancer effects of Ajwa dates in diethylnitrosamine induced hepatocellular carcinoma in Wistar rats. DEN is a strong hepatocarcinogenic substance that causes disturbances in nucleic acid repair mechanisms and also generates reactive oxygen species (ROS) leading to oxidative stress. Rats were assessed for liver cancer progression or inhibition by evaluating histological, biochemical, antioxidant enzyme status, cytokines and gene expression profiles[28]. Ghufran H, et al, 2021 & Khan F, et al, 2017; showed consistency with the current study.

The results of our study revealed that there is a significant increase in mean albumin levels and decrease in liver enzymes of ALT, AST, and GGT across experimental groups. Abd Eldaim MA study revealed that the co- or post-treatment of EST-bearing mice with GSE reduced the activities of ALT, AST, and ALP; the level AFP in serum; and hepatic P53 and PCNA protein expressions. In addition, it reduced EST-induced hepatic DNA damage and pathological alterations, while it increased serum albumin and total protein levels[29]. Yousef MI, et al, 2019; reveled that Grape seed proanthocyanidin extract reduces carboplatin and thalidomide -induced liver and heart injury throughout its potent antioxidant activity by reducing tumor suppressor gene P53, tumor necrosis factor-α, interleukin-6, liver enzymes and histological alterations in liver and heart[30]. Aldubayan MA et al, 2019; researched that thyroid hormones control the basal metabolic pace of hepatocytes. They assessed biochemical, histological and immunohistochemical changes in post pubertal hyperthyroidism and its effect on liver capacity and structures. Their results revealed, a noteworthy decrease in serum T3, T4, ALT, AST, ALP, liver MDA, P53 levels, while serum albumin, liver catalase, GSH, SOD and Bcl2 were increase in hyperthyroid mice. Treatment of hyperthyroid mice with GSPE advantages in improving the adverse effect of hyperthyroidism and also its biochemical, histopathological and P53 expression[31]. The comparative analysis with results of our results were in accordance with the findings of Abd Eldaim MA, et al, 2021, Yousef MI, et al, 2019 and Aldubayan MA et al, 2019.

Our study showed anti-cancer effects of grape seed extract on hepatocellular carcinoma by over expression of aromatase gene. Chen J, et al, 2003; conducted study on NSCLC to determined stearoyl-CoA desaturase 1 (SCD1), a crucial enzyme in lipid metabolism. SCD1 overexpression was shown to impact cell migration and invasion via aromatase (CYP19A1), an enzyme involved in estrogen production. SCD1 knockdown cells had lower levels of CYP19A1, catenin protein, and estrogen concentration. Chen J, observed the impact of SCD1 inhibitors and grape seed extract on NSCLC cells, that reduces cell migration and invasion and thus decreased tumor development and metastasis[32].Chen J, et al, 2003 showed uniformity with the results of the present study.

Our study showed anti-cancer effects of letrozole on hepatocellular carcinoma by expression of aromatase gene. Wang Y, et al, 2019; Flap endonuclease 1 (FEN1) is up-regulated by estrogen. Increased FEN1 reduces cisplatin sensitivity in breast cancer cells treatment overexpressing aromatase gene. Letrozole, an aromatase inhibitor, by suppressing the estrogen, reduced FEN1

expression while increasing cisplatin sensitivity. Thus, letrozole improves cisplatin sensitivity of breast cancer cells overexpressing aromatase via down-regulation of FEN1 in breast cancer[33]. The study of Wang Y revealed constancy with our study.

Mukherjee AG, et al, 2022; conducted a review study revealed that the increased breast cancer rate in post-menopausal women is due to highly active estrogen metabolism and ER protein expression. The high estrogen hormone production is controlled by selective estrogen receptor modulators and Aromatase inhibitors. Letrozole is an aromatase inhibitor i-e lowering estrogen synthesis, used in the treatment of adjuvant, neoadjuvant, and metastatic breast cancer, as well as in the inducement of ovulation in infertility. It also causes apoptosis, necrosis, and fibrosis, which all result in the demise of cancer cells. Mukherjee AG goes into great detail into the pharmacokinetics, pharmacodynamics, and side effects of letrozole on several organs such as the heart, kidney, liver, embryo, bone, and ovary. Certain expected or unexpected side effect due to over usage or prolonged usage than the therapeutic dose range of letrozole[34]. Letrozole given all had anti cancerous effects, however it varied in terms of pharmacokinetics, pharmacodynamics, and side effects profile. The Mukherjee AG study showed conflict with our results.

CONCLUSION

Overall, our data indicate that grape seed extract and letrozole both have impacts on liver function, as demonstrated by changes in albumin and liver enzyme levels. DEN proved to cause liver damage, but grape seed extract and letrozole may have a protective impact on liver enzyme levels and can be taken together with conventional chemotherapeutics, for synergistic benefits against HCC. Furthermore, both therapies affected the relative gene expression of the aromatase gene, indicating that they may have an impact on estrogen metabolism.

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