Comparative Histopathological & Immunohistochemical Studies between Melatonin and Grape-Seed Extract in Treating Hepatocellular Carcinoma

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Abstract: High levels of alpha- fetoprotein (AFP) are believed to be strongly suggestive of hepatocellular carcinoma (HCC) that is the fifth most frequent cancer and the third common cause of cancer related mortality in the world. AFP was studied immunohistochemically in addition to histopathology to delight the possible cure of melatonin (mel) or grape-seed extract (GSE) in induced HCC by two different carcinogens. Seventy five male albino mice were divided into six groups; normal group (n=5), experimental control group (n=10), experimental groups (n=30) and experimental treated groups (n=30). Histopathologically, the induced HCC by diethylnitrosamine (DEN) (8 w) was faster than 2-nitropropane (NP) (14 w). Malignant foci of HCC were manifested through cords of hyperchromatic malignant cells. However melatonin ameleorated these liver changes and GSE exhibited similar role but with a lesser extent. Immunohistochemically, the expression of AFP supported the superior effect of melatonin in HCC treatment. A significant value (1.20±0.77) (p=0.002)was recorded post mel treatment in comparison either with HCC (2.6±1.12) or with GSE (1.87±0.74) (p= 0.063). In conclusion: DEN induced HCC in mice faster than 2-NP and melatonin exhibited strong cure than GSE in HCC.
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1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer and the third most common cause of cancer related mortality in the world **(Jemal *et al*., 2011)**.

Alpha- fetoprotein (AFP) is a glycoprotein normally produced in large quantities during embryonic life in the foetal yolk sac and liver **(Yap & Peh 1991).** AFP is a well known representative tumor marker of HCC **(Arrigoni *et al*., 1988)**. High levels of AFP are believed to be strongly suggestive of HCC **(Tremolda *et al*., 1989)**. Greater than 70% of HCC patients have high serum concentration of AFP because of the tumor secretion. Elevation of AFP level up to pathological range in adults correlates with the appearance of several malignancies such as HCC and chronic liver disease **(Jawed *et al*., 2009)**.

Melatonin (mel.) has been demonstrated to inhibit tumor development under both *in vivo* and *in vitro* conditions **(Sirinivasam *et al.,* 2011)**. There is widespread agreement that melatonin is a useful nontoxic molecule **(Korkmaz *et al*., 2009)**.

The focus of cancer research in recent years has been shifting towards the isolation and characterization of potential chemopreventive agents present in fruits and vegetables **(Kaur *et al*., 2006)**.

Grapes (*Vitis vinifera*) are one of the most widely consumed fruits in the world **(Sharma *et al.,* 2004)**. Grape seeds extract (GSE) is a complex mixture containing gallic acid (GA), catechin (C), epicatechin (EC) and several oligomers (Procyanidins) of C and/ EC, some of which are esterified to GA **(Veluri *et al.,* 2006)**. GA is a naturally available polyphenol, possess strong antioxidant activity with a capacity to inhibit the formation of tumors in several cancer models **(Sharma *et al*., 2004)**.

GA treatment significantly reduced the levels of AFP, which revealed the anti-tumor effect of the compound against HCC **(Ramakrishnan *et al.,* 2007)**.

GSE has been shown to act as a potent scavenger of both reactive oxygen and nitrogen species. Furthermore it has also been shown that GSE significantly inhibits the activities of free-radical producing enzymes in biological systems because of its health benefits, particularly the strong antioxidant activity **(Sundaram *et al*., 2008)**.

Aim of the Work

The current study was designed to compare between the possible role of melatonin and grape-seed extract in treating HCC induced by two different carcinogens histopathologically and immunohistochemically.

2. Materials and Methods

Seventy five male albino mice, three months old (30 ± 10 g) were used in this study and divided into the following groups:

Group I: 5 mice were served as normal group.

Group II: 10 mice were served as experimental control group.

Half of them were injected I.P. with 1ml of 10mg melatonin/kg body weight every other day for 14weeks (Ravindra *et al*., 2006). The second half were received orally GSE (200 mg/kg bw/day) for 8 weeks (Singh *et al*., 2004).

Group III: 15 mice were injected I.P with 1ml of 200 mg. 2-nitropropane/kg bw every other day for 14 weeks (Borges *et al.,* 2006).

Group IV: 15 mice were injected I.P. with DEN (100 mg/kg bw 5 days/ week for 8 weeks (Subramaniyan *et al*., 2012).

Group V: 15 mice were injected with melatonin 30 minutes prior to 2- nitropropane for 14 weeks.

Group VI: 15 mice were received GSE (for 2 weeks) then (DEN) for 8 weeks.

All animals were sacrificed at the end of the experiment and the livers were prepared for the following parameters:

1- Histopathological study: sections of 5 µm were processed for H&E stains.
2- Immunohistochemical study for the detection of AFP, by using peroxidase antiperoxidase technique (Imoto *et al*., 1985).

Statistical Analysis

Data for (HCC, mel + 2-NP, and GSE + DEN) in relation to AFP intensity were expressed as mean, standard deviation (SD) and significantly using Mann Whitney test.

3. Results

Histopathological Results:

H&E stains showed the normal hepatocytes of group I with tightly packed and, pink staining cytoplasm. The nuclei were round in shape and containing prominent nucleoli, the majority of hepatocytes were mononucleated but some binucleated hepatocytes were found (Fig. 1).

Examination of liver sections of group II which received either melatonin, or GSE showed no notable alternation as compared with normal histological structure except few microsteatosis (Fig. 2) or few necrotic cells (Fig. 3) respectively.

Groups III and IV showed HCC appearance with loss of hepatic architecture and having proliferating streaks and cords of malignant hepato-cytes (Fig. 4). It is worth mentioning that there were no detectable histopathological difference between the HCC induced by 2-NP or HCC induced by DEN.

While liver sections in group V showed few large necrotic cells and microsteatosis, atypical nuclei were still seen (Fig. 5).

Group VI: liver sections showed some apoptosis and few red blood corpuscles scattered in veins, the hepatic cords were more or less regular (Fig. 6).

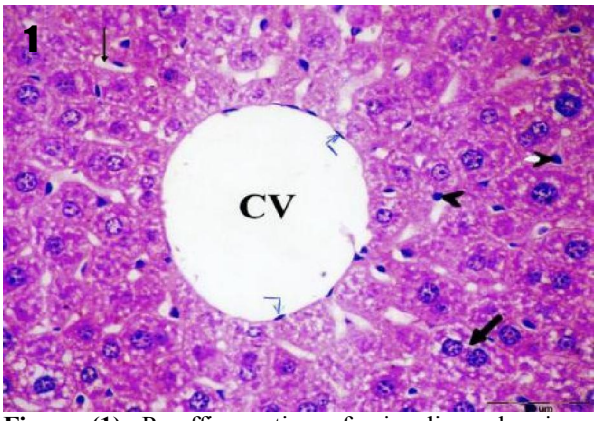

Figure (1): Paraffin section of mice liver showing normal structure. Sinusoids (→), binucleated cell (➞) Kupffer cells (➤) and central vein (CV).
H & E (bar = 50 µm).

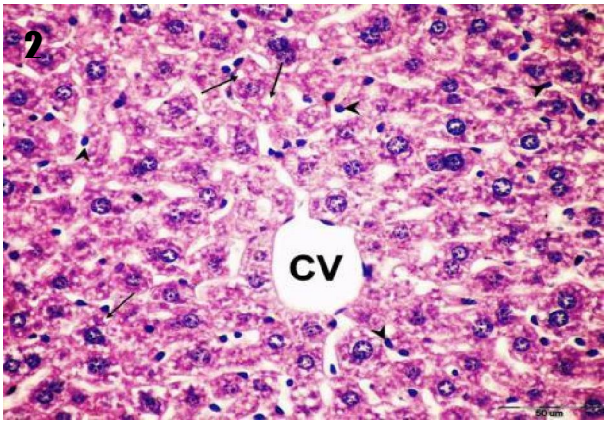

Figure (2): Paraffin section of mice liver 14 weeks post melatonin treatment showing well defined Kupffer cells (➤) and few microsteatosis (→). H & E (bar = 50 µm).

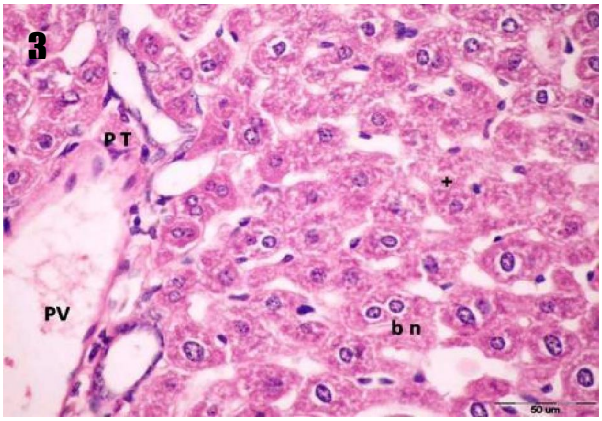

Figure (3): Paraffin section of mice liver post GSE feeding showing few necrotic cells (+), portal tract (PT), portal vein (PV) and binucleated cells (bn). H & E (bar = 50 µm).

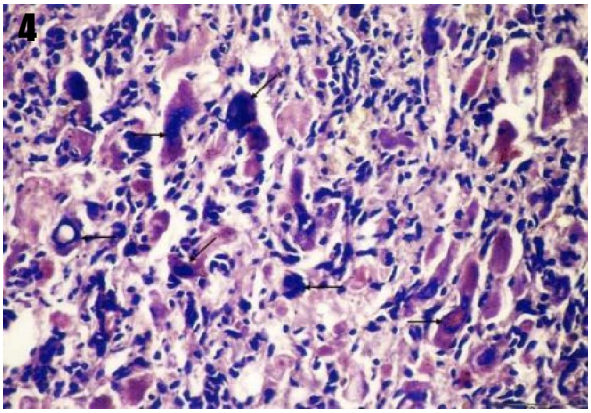

Figure (4): Paraffin section of mice liver (HCC) showing loss of hepatic architecture, proliferating streaks and cords of hyperchromatic malignant hepatocytes (→). H & E (bar = 50 µm).

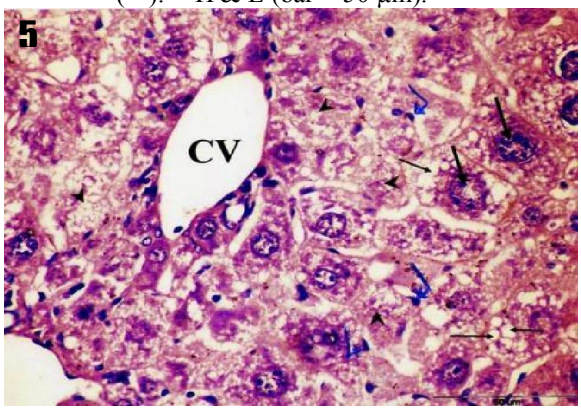

Figure (5): Paraffin section of mice liver 14 weeks post (Mel + 2- NP) showing large necrotic cells (➤), microsteatosis (→) and large atypical nuclei (→). Central vein: (CV). H & E (bar = 50 µm).

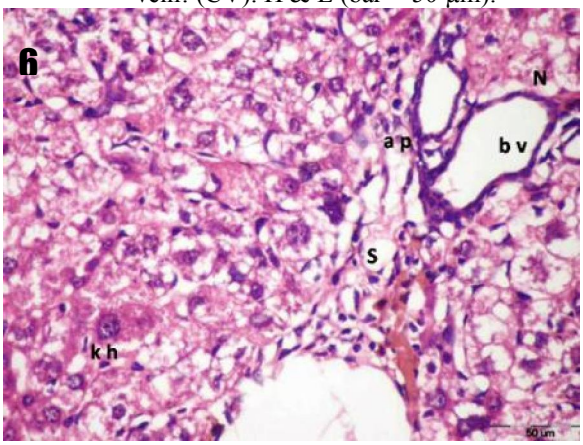

Figure (6):Liver section of mice post (GSE + DEN) showing increased necrosis (N), steatosis (S), apoptosis (ap), portal tract (PT) and portal vein (PV) and karyorrhexis (Kh). H & E (bar = 50 µm).

II. Immunohistochemical Result:

The expression of AFP is well demonstrated as brown granules in the cytoplasm and arranged around cell membranes either of hepatocytes or sinusoids.

In the present study, the normal group revealed a weak positivity of AFP (Fig. 7).

Group II: the two parts of this group showed increased positivity of AFP when compared with group I (Fig. 8).

Group III & IV: showed intense positivity of AFP in most cells (Fig. 9).

Group V: weak positivity of AFP was also seen in this group more or less as control group (Fig. 10).

Group VI: showed moderate positivity of AFP (Fig. 11).

The overall changes in AFP reaction were summarized in table (1).

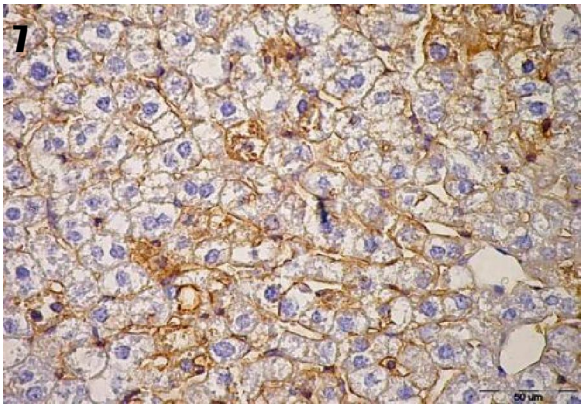

Figure (7): Paraffin section of normal control mice liver showing weak expression of AFP in most hepatic cell cytoplasm. Peroxidase antiperoxidase (PAP) method (bar = 50 µm)

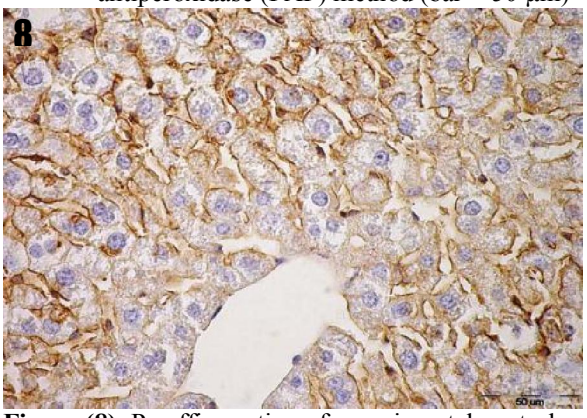

Figure (8): Paraffin section of experimental control mice liver showing increased positivity of AFP more than normal control. PAP method (bar = 50 µm)

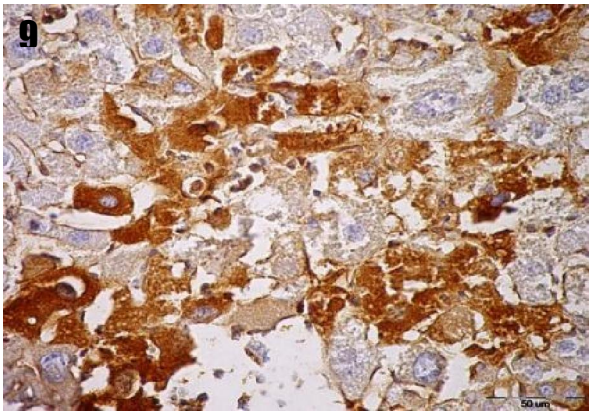

Figure (9): Paraffin section of mice liver (HCC) showing intense positivity of AFP in most cells. PAP method (bar = 50 µm)

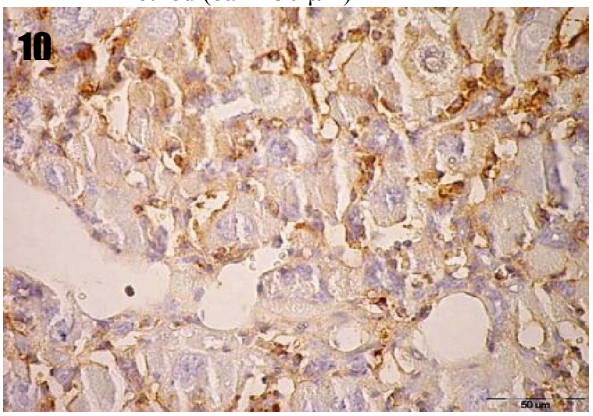

Figure (10): Paraffin section of mice liver 14 weeks post (mel. + 2 – NP) showing weak positivity of AFP more or less as control group. PAP method (bar = 50 µm)

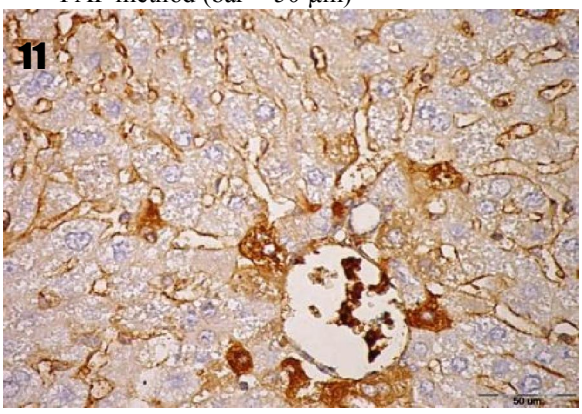

Figure (11): Liver section of mice post (GSE + DEN) showing moderate positivity of AFP. PAP method (bar = 50 µm)

Staining intensity of AFP was scored semiquantitatively on a scale of negative to intense positivity, negative (0), weakly positive (+1), moderately positive (+2), strong positive (+3) and intense positive (+4) (Table 1).

Table (2) illustrated mean and S.D of HCC group (2.60 ± 1.12), after melatonin treatment (1.20 ± 0.77) and after GSE (1.87 ± 0.74). HCC groups showed a significant increase in the AFP positivity while the experimental treated groups were significantly decreased with AFP positivity.

Table (1): Comparison between the different studied groups according to AFP

No of mice	HCC group	Mel + 2NP group	GSE + DEN group
1	+3	+2	+2
2	+1	0	+2
3	+4	+1	+1
4	+3	0	+1
5	+4	+1	+2
6	+4	+2	+1
7	+2	+1	+2
8	+2	+1	+2
9	+3	0	+3
10	+4	+2	+3
11	+3	+1	+1
12	+2	+2	+2
13	+1	+2	+3
14	+2	+2	+1
15	+1	+1	+2
Min.	1.0	0.0	1.0
Max.	4.0	2.0	3.0
Mean	2.60	1.20	1.87
±SD	1.12	0.77	0.74
Median	3.0	1.0	2.0

Table (2): Comparison between the different studied groups according to AFP

Intensity of AFP	HCC group		Mel + 2NP group		GSE + DEN group	
	No.	%	No.	%	No.	%
0	0	0.0	3	20.0	0	0.0
+1	3	20.0	6	40.0	5	33.3
+2	4	26.7	6	40.0	7	46.7
+3	4	26.7	0	0.0	3	20.0
+4	4	26.7	0	0.0	0	0.0
Min. – Max.	1.0 – 4.0		0.0 – 2.0		1.0 – 3.0	
Mean ± SD	2.60 ± 1.12		1.20 ± 0.77		1.87 ± 0.74	
Median	3.0		1.0		2.0	
p			0.002*		0.063	

p: *p* value for Mann Whitney test
*: Statistically significant at $p \leq 0.05$

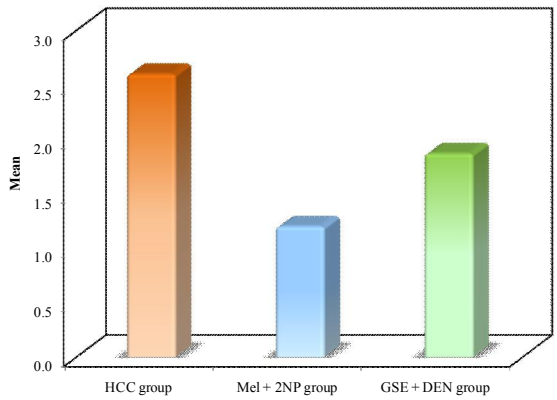

Figure (12): Comparison between the different studied groups according to AFP

These results were expressed as mean ±SD, $p \leq 0.05$.

4. Discussion

There are several mechanisms by which melatonin can exert its oncostatic actions by its: direct pro-apoptotic, gene-mediated actions on tumor cells, antioxidant actions, reducing the uptake of key factors of tumor growth and tumor growth signaling molecules e.g. linoleic acid and by enhancing immune mechanisms in the body.

Our present results illustrated that melatonin improved the histological feature of hepatocytes when administered 30 min. prior to 2-NP. More or less GSE, with little degree, made the same improvement when administered prior to DEN.

Our present findings were coincided with Dakshayani *et al*. (2005) who indicated that melatonin exerts a chemo- preventive effect. They demonstrated that melatonin restored the activities of hepatic marker enzymes and reversed the oxidant antioxidant imbalance during N-nitroso-diethylamine- induced hepatocarcinogenesis.

In accordance, Fan *et al.* (2010) showed that the synergism of melatonin and doxorubicin inhibited hepatoma cell growth and induced cell apoptosis.

Growth inhibition by melatonin altered the percentage of cells in G0-G1 and G2/M phases indicating cell cycle arrest in the G2/M phase. (Martin *et al*., 2005).

Zha *et al.* (2012) demonstrated that melatonin sensitized human hepatoma cells to endoplasmic reticulum stress-induced apoptosis by down-regulating COX-2 expression, increasing the levels of CHOP (pro-apoptotic transcript factor) and decreasing the BCL-2/ Bax ratio.

These changes in the cell cycle depend on the melatonin dose administration to the HepG2 tumor cells (Martin *et al*., 2008).

These novel findings show that melatonin by inducing cell death and cell cycle arrest, might be useful as adjuvant in hepatocarcinoma therapy (Martin *et al*., 2008).

Regarding GSE many studies have shown that it inhibited human breast carcinoma MCF-7, human lung cancer A-427 and human gastric cancer cell CRL-1739 growth (Veluri *et al*., 2006). Another studies also showed that, GSE inhibited cell proliferation, induced cell cycle arrest and induced apoptotic cycle in human breast carcinoma cells MDA-MB468 and prostate Du 145 carcinoma cells (Agarwal *et al*., 2000 and Agarwal *et al*., 2002). Several studies in mice showed that GSE inhibited chemically – induced lipid peroxidation, DNA fragmentation, and subsequent apoptosis (indicators of oxidative tissue damage) in a dose-dependent manner in hepatic and brain tissues (Bagchi *et al*., 1998).

Respecting AFP, there was shortage in literature which dealing with its immunohistochemical expression in HCC under the effect of mel. and perhaps, the present study may be the first one in this field.

Our present results showed that AFP was re-expressed in HCC induced by 2-NP while after melatonin treatment its concentration was weak (more or less as control group). In accordance to GSE treatment this positivity was moderate.

In accordance with our findings, (Ray *et al*., 2000) Reported that GSE administration was beneficial in preventing animal hepatic and renal toxicity. These authors studied the role of GSE in acetaminophene and other drug poisoning and attributed the protective effect of GSE to detoxification of cytotoxic free radicals or facilitation of DNA repair. Our results supported the role of phenolics to attenuate the alterations resulted through HCC induction by DEN. (Sundaram *et al*., 2008), (Kahkonen *et al.,* 1999) The antioxidant activity of these compounds may be mainly due to their redox properties that allow them to act as reducing agents,hydrogen donators, and singlet oxygen quenchers. In conclusion: DEN induced HCC in mice faster than 2-NP and melatonin exhibited strong cure than GSE in HCC.

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References:

1. Agarwal C, Rana P, Singh RP, Agarwal R. Grape seed extract induces apoptotic death of human prostate

carcinoma DU145 cells via caspases activation accompanied by dissipation of mitochondrial membrane potential and cytochrome c release. Carcinogenesis 2002; 23 (11): 1869-1876.
2. Agarwal C, Sharma Y, Zhao J, Agarwal R. A polyphenolic fraction from grape seeds causes irreversible growth inhibition of breast carcinoma MDA-MB 468 cells by inhibiting mitogen-activated protein kinases activation and inducing G1 arrest and differentiation. Clin Cancer Res 2000; 6: 2921-2930.
3. Arrigoni A, Andriulli A, Gindro T, Piantino P, Capussotti L, Rizzetto M. Pattern analysis of serum alpha-fetoprotein in the early diagnosis of hepatocellular carcinoma in liver cirrhosis. Int J Biol Markers 1988; 3 (3): 172-6.
4. Bagchi D, Garg A, Krohn RL. Protective effects of grape seed proanthocyanidins and selected antioxidants against TPA-induced hepatic and brain lipid peroxidation and DNA fragmentation, and peritoneal macrophage activation in mice. Gen Pharmacol 1998; 30: 771-776.
5. Borges LP, Nogueira CW, Panatieri RB, Rocha JB, Zeni G. Acute liver damage induced by 2-nitropropane in rats: Effect of of diphenyl diselenide on antioxidant defenses. Chem Biol Interact 2006; 160:99-107.
6. Dakshayani KB, Subramanian P, Manivasagam T, Essa MM, Manoharan S. Melatonin modulates the oxidant-antioxidant imbalance during N-nitrosodiethylamine induced hepatocarcinogenesis in rats. J Pharm Pharm Sci 2005; 8 (2): 316-321.
7. Fan LL, Sun GP, Wei W, Wang ZG, Ge L, Fu WZ, Wang H. Melatonin and doxorubicin synergistically induce cell apoptosis in human hepatoma cell lines. World J Gastroenterol 2010; 16 (12): 1473-1481.
8. Imoto M, Nishimura S, Fukuda Y, Sugiyama K, Kumada T, Nakano S. Immunohistochemical detection of alphafetoprotein, carcinoembryonic antigen and ferritin in formalin-fixed paraffin sections from hepato-cellular carcinoma. Am J Gastro-enterol 1985; 80:902-906.
9. Jawed AB, Junaid MA, Syed RM, Muhammad B, Rabia S, Ishrat S, Abdul Waheed. Hepptocellular Carcinoma (HCC) and Diagnostic Significance of alpha-Fetoprotein (AFP). J Ayub Med Coll Abbottabad 2009; 21(1): 72-75.
10. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61:69
11. Kahkonen MP, Hopia Al, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS. Antioxidant activity of plant extracts containing phenolic compounds. J Agric Food Chem 1999; 47: 3954- 3962.
12. Kaur M, Rana P, Singh MG, Agarwal R, Agarwal C. Grape Seed Extract Inhibits *In vitro* and *In vivo* Growth of Human Colorectal Carcinoma Cells. Clin Cancer Res 2006; 12 (20): 6194-6202.
13. Korkmaz A, Reiter RJ, Topal T, Manchester LC, Oter S, Tan D. Melatonin: an established antioxidant worthy of use in clinical trials. Mol Med 2009; 15: 43-50.
14. Martin-Renedo J, Mauriz JL, Jorquera F,Ruiz-Andres O, Gonzalez P, Gonzalez-Gallego J. Melatonin induces cell cycle arrest and apoptosis in hepatocarcinoma HepG2 cell line. J Pineal Res 2008; 45: 532-540.
15. Ramakrishnan G, Augustine TA, Jagan S, Vinodhkumar R, Devaki T. Effect of silymarin on N-nitrosodiethylamine induced hepatocarcinogenesis in rats. Exp Oncol 2007; 29 (1): 39-44.
16. Ravindra T, Lashmi NK, Ahuja YR. Melatonin in pathogenesis and therapy of cancer. Ind J Med Sci 2006; 60:523-535.
17. Ray SD, Patel D, Wong V, Bagchi D. *In vivo* protection of DNA damage associated apoptotic and necrotic cell deaths during acetaminophen induced nephrotoxicity, amiodarone-induced lung toxicity, and doxorubicin-induced cardiotoxicity by a novel IH636 grape seed proanthocyanidin extract. Res Commun Mol Pathol Pharmacol 2000; 107: 137-166.
18. Yap SF, Peh SC. Alpha-Fetoprotein in Hepatocellular Carcinoma: A serologlcal and Histochemical Study in Malaysian Patients Malaysian. J Pathol 1991;13(2): 115-118.
19. Sharma G, Tyagi AK, Singh RP, Daniel CF, Chan, Agarwal R. Synergistic anti-cancer effects of grape seed extract and conventional cytotoxic agent doxorubicin against human breast carcinoma cells. Breast Cancer Research and Treatment 2004; 85: 1-12.
20. Singh RP, Tyagi AK, Sivanandhan DS, Agrwal R, Agrwal C. Grape seed extract inhibits advanced human prostate tumor growth and angiogenesis and upregulates insulin-like growth factor binding protein-3. Int J Cancer 2004; 108: 733-40.
21. Srinivasan V, Pandi-Perumal SR, Brzezinski A, Bhatnagar KP, Cardinali DP. Melatonin, immune function and cancer. Metabolic & Immune Drug Discovery 2011; 5(2) 109-123.
22. Subramaniyan J, Arumugane M, Selvamani A. Potential preventive effect of carvacrol against diethylnitrosamine - induced HCC in rats. Mol Cell Bioch 2012; 360 (1-2): 51-60.
23. Sundaram J, Gopalakrishnan R, Pandi A, Sattu K, Thiruvengadam D. Antiproliferative potential of gallic acid against diethylnitrosamine-induced rat hepatocellular carcinoma. Mol Cell Biochem 2008; 319: 51-59.
24. Tremolda F, Benevegnu L, Drago C. Early detection of hepatocellular carcinoma in patients with cirrhosis by alphafetoprotein, ultrasound and fine-needle biopsy. Hepato-gastroenterology 1989; 36 (6): 519-21.
25. Veluri R, Singh RP, Liu Z, John A, Thompson JA, Agarwal R, Agarwal C. Fractionation of grape seed extract and identification of gallic acid as one of the major active constituents causing growth nhibition and apoptotic death of DU145 human prostate carcinoma cells. Carcinogenesis 2006; 27 (7): 1445-1453.
26. Zha L, Fan L, Sun G, Wang H, Ma T, Zhong F, Wei W. Melatonin sensitizes human hepatoma cells to endoplasmic reticulum stress-induced apoptosis. J Pineal Res 2012; 52(3): 322-31.

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