

Synergistic Anticancer Activity of *Terminalia Muelleri* and Doxorubicin on Chemically Induced HCC in Albino Rats

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Abstract: The present study aimed to evaluate the curative role of *Terminalia muelleri* ethanolic extract (TME) against diethyl nitrosamine (DENA) induced HCC, in addition to evaluate its role as a combined therapy with Doxorubicin (DOX) which is one of the standard chemotherapy. Sixty adult male albino rats were divided into sixteen control rats and forty-four rats injected with DENA to induce HCC. The induction of HCC was manifested through the significant increase in the enzymatic activity of serum aminotransferases (ALT and AST) with sever changes in liver architecture and HCC was confirmed by the significant increase in serum AFP. The progression of HCC was evidenced by the significant increase in serum AFP, decrease in serum albumin levels and increase proliferation of hepatocellular carcinoma in association with significant decrease of caspase-3 immunoreactivity. HCC-induced rats were subjected to the treatment with DOX, TME and the combination of both. Normal rats were divided into control non-treated group and TME administered group. The obtained data proved that, the treatment with TME elicited an efficient curative effect in the treatment of HCC in comparable with DOX towards HCC and the combination of both exerted a more pronounced curative effect than that obtained by the individual treatment. In conclusion TME could be a promising or adjuvant therapy in the treatment of HCC.

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I. Introduction

Hepatocellular carcinoma (HCC) represents 70–85% of cases of liver cancer. It accounts the fifth utmost common cause of cancer and the third cause of cancer-associated deaths worldwide.^[1]

In Egypt, HCC participates to 14.8% of all cancer mortality. HCC is the second utmost frequent cancer type in Egyptian males after bladder cancer and the eighth utmost frequent in Egyptian females.^[2] HCC is a significant reason of morbidity and mortality and have an unfavorable prognosis with aggressive growth activity and a high rate of recurrence.^[3]

Xenobiotics are possibly hepatotoxic. The ability of a chemical to generate liver damage *in-vivo* often causes the interaction of a sequences of complex cellular processes which are involving in the uptake, biotransformation and removal of these potentially toxic components.^[4]

Doxorubicin comprises of a three-aromatic ring-based molecule (also called Adriamycin) relates to a class of compounds with identical structures called anthracyclines. Doxorubicin was separated from *Streptomyces peucetius* (a soil bacterium).^[5] Doxorubicin is one of the primary chemotherapy drugs utilized for HCC and exhibited interesting results.^[6] Chemotherapy for cancer treatment utilizes drugs are called cytostatic that aims to terminate cancer cells from enduring to divide uncontrollably.^[7]

Natural ingredients have lengthy been a source of anti-tumor compounds.^[8-10] Herbal drugs are commonly low in cost, considerable and demonstrate very little toxicity or adverse effects in clinical practice.^[11,12] Epidemiological studies propose that ingesting of diets containing fruits and vegetables that are the major sources of phytochemicals, micronutrients and macronutrients share in decreasing the risk of cancer development.^[13]

The genus *Terminalia* are widely used in traditional medicine in several continents in the world for the treatment of numerous diseases including, abdominal disorders, bacterial infections, colds, sore throats, conjunctivitis, diarrhea, dysentery, fever, gastric ulcers, headaches, heart diseases, hookworm, hypertension, jaundice, leprosy, nosebleed, oedema, pneumonia and skin diseases.^[14, 15]

In our previous study we demonstrated that, the investigation of ethyl acetate extracts of two different species of *Terminalia* by HPLC-UV-MS/MS-ESI revealed the following flavonoid compounds: Rutin, quercetin, methyl gallate and caffoyl acetyl hexoside in ethyl acetate fraction of *T. muelleri* leaf. Rutin, quercetin, epigallocatechin gallate and myricetin in ethyl acetate fraction of *T. myriocarpa* leaf. The quantitative

determination of these identified compounds, rutin and quercetin in ethyl acetate fraction of both plants by LC-MS/MS revealed that quercetin was the major (20.53, 108.60 µg/mg ethyl acetate) followed by rutin (1.18, 81.83 µg/mg ethyl acetate) of *T. muelleri* and *T. myriocarpa* leaf, respectively.^[16] Our previous work also demonstrated the role of *Terminalia muelleri* in the protection of liver tissue against alcohol induced liver damage.^[17] The present work was the first to evaluate the therapeutic role of *Terminalia muelleri* in the form of ethanolic extract against DENA-induced HCC. In addition to evaluate its role as a combined therapy with Doxorubicin. Animal models can be used to mimic human carcinogenesis and the development of HCC.^[18] DENA is one of the most potent hepatocarcinogenic agent^[1]

II. Materials and methods

Materials

Terminalia muelleri (TM) Collection

The leaves of *Terminalia muelleri* were collected in the month of July to September from Giza zoo and authenticated by Dr. Traze, Botanist, Orman garden and had been deposited in the Department of Biochemistry, NODCAR.

Chemicals

Doxorubicin hydrochloride (**DOX**) was purchased from Eimic Company, Cairo, Egypt. Diethyl nitrosamine (**DENA**) was purchased from Sigma– Aldrich (St. Louis, USA) and stored in a light-protected container at 4°C.

All chemicals of pure analytical grade and were purchased from Sigma– Aldrich (USA), Fluka (Switzerland) and British Drug House (BDH, England). **Experimental animals**

Sixty male albino rats, weighing 150-200g were obtained from Animal house, Research institute of Ophthalmology, Giza, Egypt. All rats were maintained in an air-conditioned animal house with specific pathogen free conditions and were subjected to a 12:12-h day light/darkness cycle. The animals were allowed free access to water and rats were fed on the standard diet that was composed of a mixture of 72.2% carbohydrate, 3.4% fats, 19.8% proteins, 3.6% cellulose, 0.5% vitamins and minerals and 0.5% salts obtained from Kahira Company for Poultries, Cairo, Egypt. Rats were kept at constant environmental and nutritional conditions through period of experiment. The investigation was complied with the Guide for Care and Use of Laboratory Animals published by the US national institutes of health.

Methods

Preparation of *T. muelleri* ethanolic extract (TME)

The leaves were shade dried and made core powder. The powder was then packed into Soxhlet apparatus and subjected to continuous percolation using ethanol (95% v/v) as a solvent. The extract was concentrated under vacuum evaporator.^[19] Extract was stored at 4 °C and protected from light and humidity in vacuum desiccator until further use. The extract was a dark green solid.

Preparation of Chemotherapeutic drugs (Doxorubicin, DOX)

Doxorubicin was prepared and diluted under sterile conditions in 0.9% saline.

Experimental design

Induction of hepatocellular carcinoma

Forty-four rats were intraperitoneally injected with DENA (10 mg/kg b. wt. that was diluted in sterile 0.9% sodium chloride solution) for five consecutive days for 6 weeks.^[20] Blood samples were withdrawn after 6 weeks to assess the induction of liver injury by measuring the activities of ALT and AST in the serum as well as histopathological examination for liver samples. Serum AFP was examined to confirm the induction of HCC in DENA challenged rats. Sixteen healthy rats were employed simultaneously and served as control groups.

Treatment Period

HCC-induced rats were divided into four groups (8 rats for each) as follows:

- DENA group or positive control group (DENA group).
- HCC-induced rats were IP injected with DOX (1mg/kg b.wt.) for three alternative days/week for 8 weeks.^[21] This group served as DENA+ DOX group.
- HCC-induced rats were administered with a daily oral dose of *T. muelleri* ethanolic extract in 1 % gum acacia (100 mg /kg b.wt) for 8 weeks.^[19] This group served as DENA+ TME group.
- HCC-induced rats were IP treated with doxorubicin concomitantly with the oral administration of *T. muelleri* ethanolic extract for 8 weeks. This group served as DENA+ DOX+ TME group.

Control groups classification

Control groups were composed of (8 rats for each) divided into two groups:

- Normal control group: rats of this group were administered a daily oral dose of 1% gum acacia vehicle (1 ml of 1% gum acacia) for 8 weeks.
- Plant group: rats of this group were administered a daily oral dose of *T. muelleri* ethanolic extract in 1% of gum acacia (100 mg/kg b.wt.) for 8 weeks.^[19]

Sampling

At the end of the treatment period (8 weeks), blood samples were collected for serum separation in centrifuged tubes by ocular vein puncture. Tubes were centrifuged at 2000 rpm for 15 minutes at 4 °C. The separated serum samples were stored at -20°C for biochemical analysis. Liver tissues were excised rapidly and used for histological examination

Serum biochemical parameters

Serum *alanine aminotransferase* (ALT) and *aspartate aminotransferase* (AST) activities were determined according to the method of **Reitman and Frankle**^[22] using kits provided by Quimica Clinica aplicada (QCA, Spain). Serum albumin (ALB) was determined according to the method **Doumas, et al.**^[23] using kits provided by Biomerieux (France). Alpha fetoprotein (AFP) was determined using rat alpha-fetoprotein (AFP) ELISA kit (My BioSource, San Diego, California) according to the manufacturer's instructions.

Histological examination

Samples of liver tissue were taken from all groups and fixed in 10% formalin for twenty-four hours. Washing was done in tap water then serial dilutions of alcohols (methyl and ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty-four hours. Paraffin bees- wax tissue blocks were prepared for sectioning at 4μ thickness by slide microtome. The obtained tissue sections were collected on glass slides, deparaffinized, stained by haematoxylin and eosin (H and E) stain for examination through the light electric microscope.^[24]

Immunohistochemistry technique

Proliferating cell nuclear antigen (PCNA)

Immunohistochemistry staining for PCNA to detect cell proliferation was performed on fixed, paraffin-embedded sections from rat liver tissue. The primary antibody employed was the anti-PCNA monoclonal antibody PC-10 for rats supplied by **Triology, (Sigma Aldrich, USA)** according to **Omata et al.**^[25]

Caspase-3

Immunohistochemistry staining for caspase-3 to detect cell apoptosis was performed on paraffin embedded liver tissues, using specific anti-sera containing primary antibody for rats supplied by **Abbkine, Inc, (China)** according to **Bajracharya et al.**^[26]

Then slides were examined under binocular light microscope, photographed used digital image capture system (**Olympus, Tokyo, Japan**) and the intensity of the immunostaining of the hepatocytes and biliary epithelium were graded as negative weak, moderate, or strong. All stained slides of all groups were photographed used digital image capture system (**Olympus CX40, Tokyo, Japan**) using software program (**Image pro-plus version 5**).

Immunohistochemistry evaluation

The ordinary light microscope was used to detect and examined by an image Analyzer computer system using the software (image pro-plus version 5). Six random fields in area percentage of positive cells. Data obtained as mean area% and standard error (mean of area% ± SE).

Statistical analysis

The results were expressed as the mean values ± standard error of the mean (mean± SE). Difference between the control group and HCC-induced rats was assessed using independent t-test. Variance between control, HCC-induced rats and treated groups was assessed using one-way analysis of variance (ANOVA) at p< 0.05. Subsequent multiple comparisons between the different groups were analyzed by Duncan's multiple comparisons test for the assumption of homogeneous variance and Games Howell for the assumption of non-homogeneous of variance at p 0.05. Data were statistically analyzed using the statistical package of social science (SPSS) version 23.

III Results

The assessment of hepatocellular carcinoma (HCC) induction by DENA

ALT and AST enzymatic activity was performed as a monitor for the assessment of hepatocellular damage. Table 1 shows that the IP injection of DENA (10 mg/Kg b.wt.) for five consecutive days for six weeks caused a well-marked elevation ($p<0.05$) in the enzymatic activity of serum ALT and AST compared to control non-treated group. The magnitude of this increase is 300% and 255.1%.

Table 1: Effect of DENA on serum AST and ALT enzymatic activity

Groups \ Parameters	AST (U/ml)	ALT (U/ml)
Control	24.5 ± 0.36	25.3 ± 0.38
DENA	$97.9 \pm 1.18^{***}$	$89.82 \pm 1.81^{***}$
% Change	300. 0%	255.1%

Data are expressed as mean \pm SE (n = 60 rats; 16 rats for control and 44 rats for DENA-treated rats). ***means very highly significant difference between DENA-injected rats and control non-treated rats at $p<0.001$. Difference between groups was assessed using independent t-test. % change was calculated with respect to control non-treated group

Figure 1 (A) shows the normal liver architecture. The microscopical examination of liver tissue of DENA injected rats revealed that, the IP injection of DENA for six weeks resulted in dilatation of few portal vein with multiple newly formed bile ductules with oedema and few inflammatory cells infiltration surrounding by hepatocyte. Hepatic tissues of this group showed polarity and pleomorphism of the anaplastic hepatocytes, karyomegalocytes with prominent nucleoli of the anaplastic hepatocyte which reflected the induction of HCC Fig. 1 (B and C).

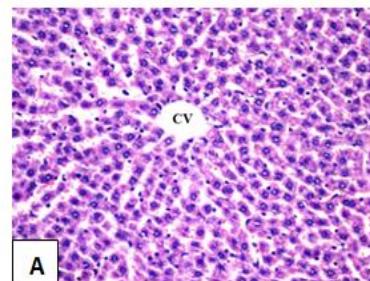


Fig. 1 (A): Control non-treated group (H&E) X 100

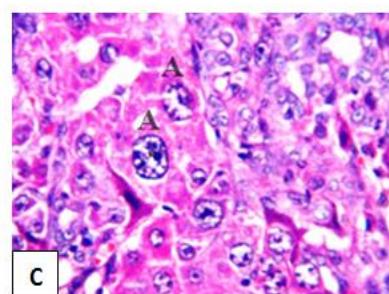
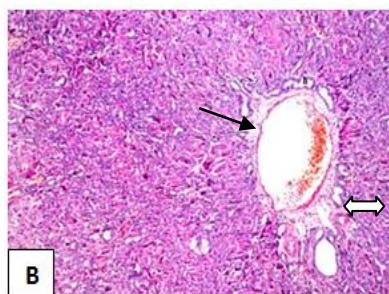


Fig. 1(B, C): photomicrograph of hepatic tissue of DENA treated group showing: proliferated bile duct canaliculi (double arrow) and oedema (arrow) and A: anaplastic cells (H&E) X 400

To confirm the induction of HCC, serum AFP level was measured. The obtained data revealed that IP injection of DENA for six weeks caused a significant increase in serum AFP level. The magnitude of this increase is 256% with respect to control non-treated group Fig. 2.

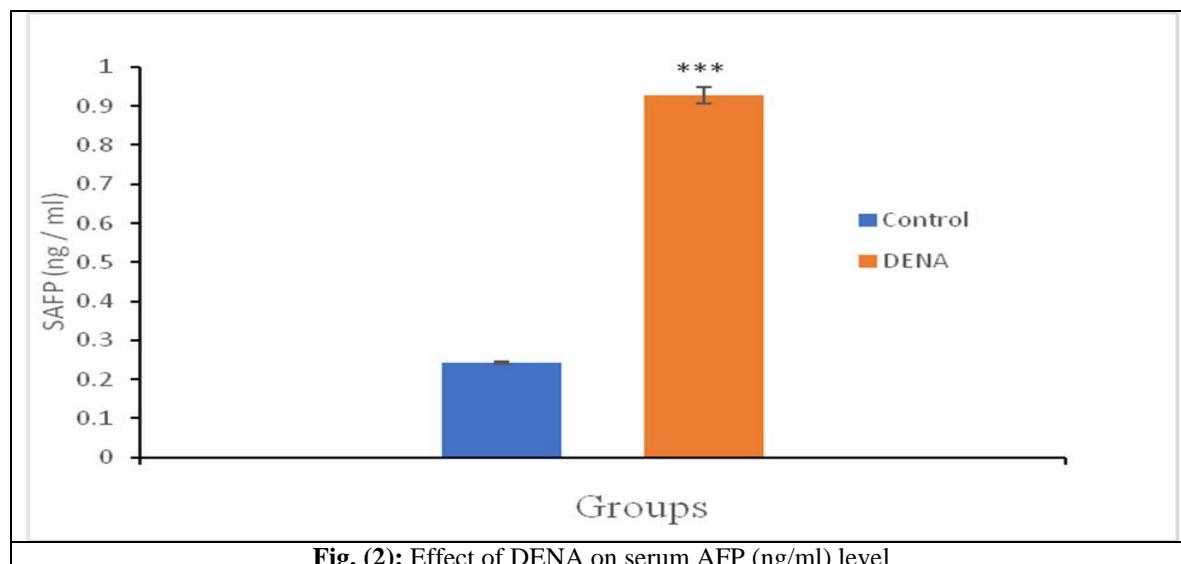


Fig. (2): Effect of DENA on serum AFP (ng/ml) level

Data are expressed as mean \pm SE ($n = 60$ rats; 16 rats for control and 44 rats for DENA-treated rats). ***means very highly means significant difference between DENA-injected rats and control non-treated rats at $p < 0.001$. Difference between groups was assessed using independent t-test

The curative effect of DOX, TME and their combination on DENA-induced HCC on the following parameters

Serum level of Alpha fetoprotein (SAFP)

Table 2 shows that, the sole administration of TM ethanolic extract (TME) induced no change in the level of SAFP with respect to the control non-treated groups. The obtained data also revealed that, DENA-injected rats displayed a well-marked progressive increase in SAFP level at the end of experimental period when compared with the group of rats injected by DENA for the first six weeks to induce HCC (Fig. 2).

Table 2 also shows that, the sole administration of DOX and TME to DENA-challenged rats caused a significant decrement in SAFP levels when statistically compared with DENA-challenged rats. The magnitude of this decrease with respect to DENA-challenged rats is -54.1% and -62.4% in SAFP levels for DOX and TME respectively. The concomitant administration of TME with DOX caused a well-marked reduction in SAFP level with 78.05% decrease when compared with DENA-challenged rats as well as TME increases the therapeutic effect of DOX to induce a 2-fold improve in SAFP level with respect to the group of rats treated with DOX alone (Table 2).

Serum Albumin (SALB)

Albumin was employed in the present study to monitor the synthetic function of liver tissue. Table 2 shows that, the administration of TME to healthy rats induced no significant changes in SALB levels when statistically compared with the control non-treated groups.

A significant reduction in SALB level was recorded in DENA injected rats with respect to the control non-treated groups (Table 2). Data shown in the Table revealed that, treatment of DENA-challenged rats with DOX caused a 13.05 % significant increase in SALB level with respect to DENA-injected rats, meanwhile the treatment of DENA-injected rats with TME recorded a marked increase ($p < 0.05$) in SALB Level when compared with either DENA-injected rats or DOX treated rats, the magnitude of this increment is 54.8% with respect to DENA-injected rats. The co-administration of DOX with TME significantly improved the level of SALB with 45.1% increase with respect to DENA-injected rats. The obtained data also showed that TME increased the therapeutic action of DOX by inducing 28.4% increase in ALB level when compared with the group of rats treated with DOX alone (Table 2).

Table 2: The effect of DOX, TME and their combination on serum levels of Alpha fetoprotein (SAFP) and Albumin (SALB)

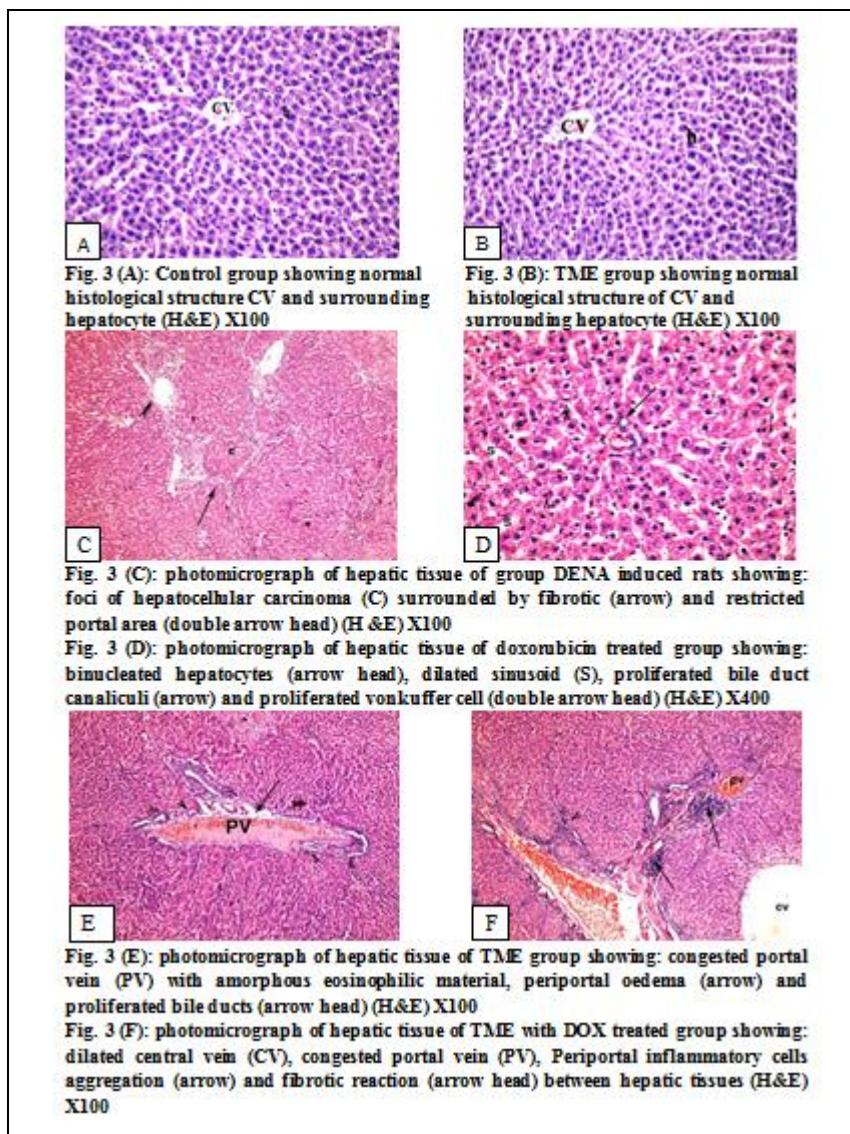
parameters Groups	AFP (ng /ml)	ALB (g /dl)
Control	0.27 ± 0.01 ^A	4.29 ± 0.08 ^E
TME	0.29 ± 0.01 ^A	4.26 ± 0.08 ^E
DENA	2.05±0.09 ^E	2.54 ± 0.10 ^A
DENA + DOX	0.94 ± 0.02 ^D	2.87 ± 0.09 ^B
DENA + TME	0.77 ± 0.02 ^C	3.94 ± 0.04 ^C
DENA+DOX+ TME	0.45 ± 0.01 ^B	3.66 ± 0.07 ^D

Values are mean ± SE (n= 6). In the same column, the presence of different superscript means a significant difference between groups. ANOVA test followed by Games Howell multiple comparisons between groups at P< 0.05 for SAFP and Duncan's multiple comparisons between groups at P< 0.05 for SALB

Histopathological investigations

The illustrative photomicrographs of hepatic tissues of control group (Fig. 3A) or TME-treated group (Fig. 3B) showed normal histological architecture of CV and surrounding hepatocytes.

Meanwhile, the demonstrative photomicrographs of hepatic sections attained from animals in DENA-treated group displayed the proliferation of fibroblastic cells was extended from the portal area between the degenerated hepatocytes dividing the hepatocytes into lobules (Fig. 3C) with cystic dilatation and neoplastic activation of the bile ducts forming cystic cholangioma. Hepatocytes displayed vacuolated cytoplasm, marginated nucleus and hyperchromatic activity. Also, foci of HCC and necrotic areas were noticed. The treatment of DENA-induced HCC rats receiving Doxorubicin showed clear focus of hepatocytes with vacuolar cytoplasm and pyknotic nuclei in the hepatocytes and act as precursor of neoplasia. The portal areas showed oedema with hyperplasia in the bile ducts. Mild necrotic areas and proliferated vonkuffer cell were observed (Fig.3 D). Moreover, DENA-treated rats with TME showed moderate histopathological changes in the vasculature where central vein displayed dilatation and expansion of portal vein with periportal oedema and proliferated bile ducts. Hepatocellular carcinoma was clearly indicated (Fig. 3E). The architecture of hepatic tissue of DENA-treated rats receiving the combination therapy of TME and DOX showed dilated central vein, portal vein congestion in addition to fibrotic reaction between hepatic tissue. This combination therapy could cause the regenerative reaction with diffuse binucleated hepatocytes coupled with normal bile ducts (Fig. 3F).



PCNA under the influence of DENA-induced HCC

To evaluate the anticancer activity of TME, the proliferating cell nuclear antigen (PCNA) protein was performed. In the present study PCNA was almost absent in control non-treated and TME groups (Fig 4A and 4B). Meanwhile, DENA-treated group showed a strong positive staining for PCNA indicating active proliferation of liver tissue (Fig. 4C). Figures 4D and 4E show that the sole administration of DOX and TME to HCC rats lowered PCNA expression in the following manner TME<DOX.

Figure (4F) shows that, the combined administration of TME with DOX improved the antiproliferative effect of DOX. Figure (4G) depicts the area % of the immunohistochemical staining expression levels of PCNA in liver sections of different groups.

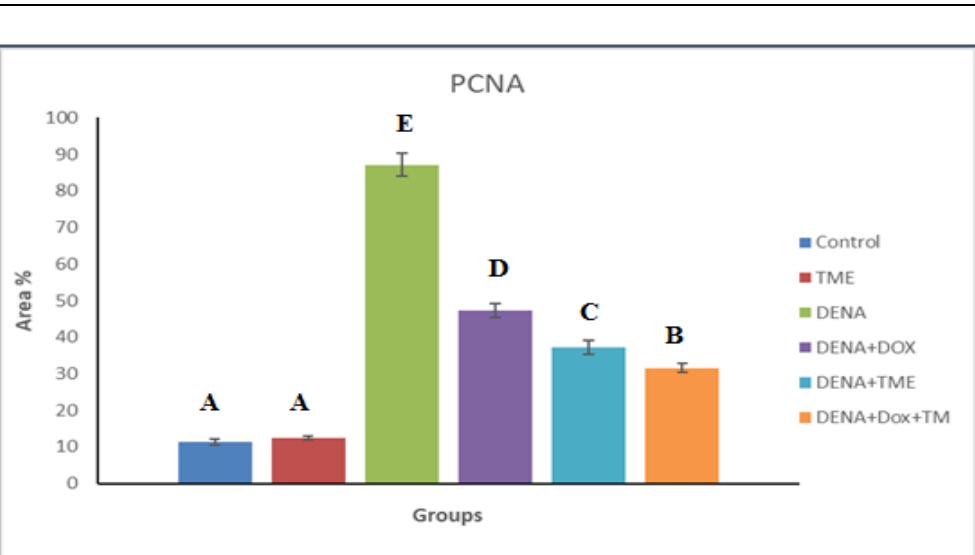
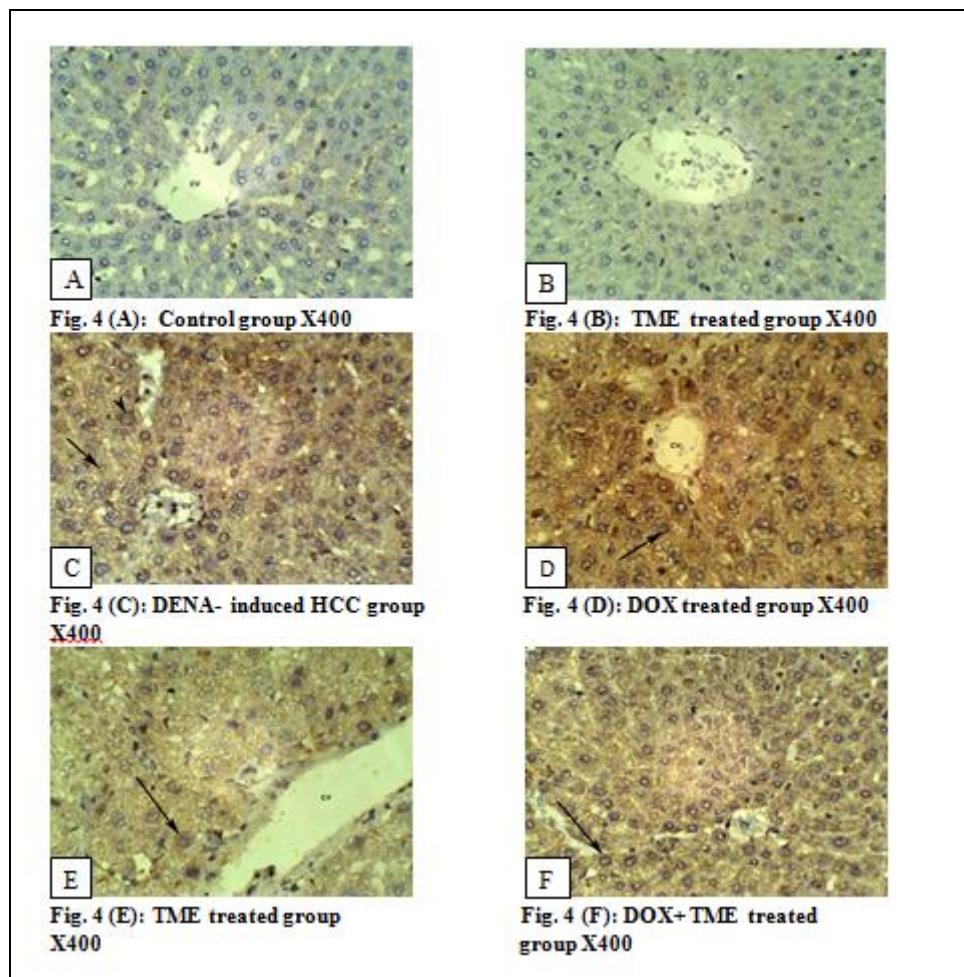
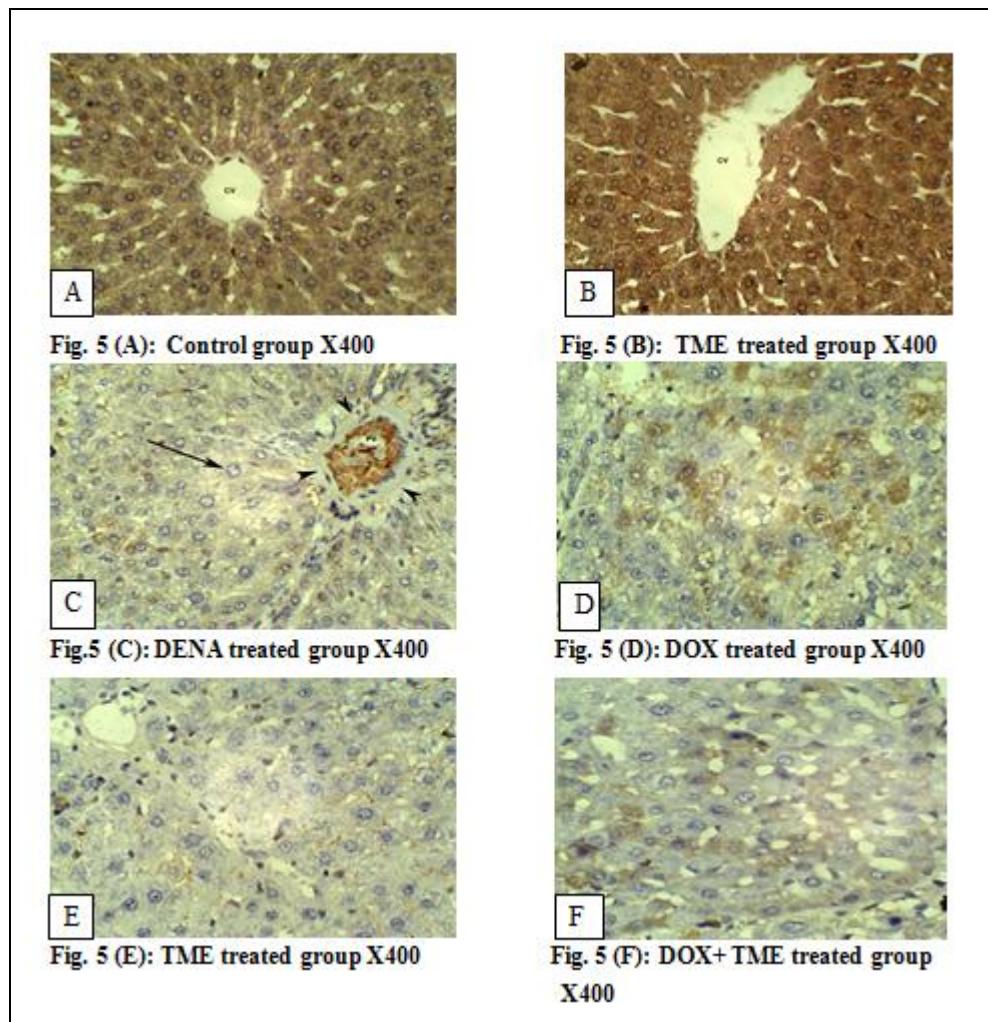


Fig. 4 (G): The mean of area % of PCNA in control groups and under the influence of different treatments

Values are mean \pm SE ($n= 6$). The presence of different letter on each bar means a significant difference between groups. ANOVA test followed by Duncan's multiple comparisons between groups at $P < 0.05$ were employed

Caspase-3 under the influence of DENA-induced HCC:

Figures 5A and 5B show the expression level of Caspase-3 in control non- treated rats and the group of normal rats administered TME alone. A significant decrease in caspase-3 expression level is observed in the group of rats treated with DENA (Fig. 5C). Figures 5D and 5E show that the sole administration of DOX and TME to HCC rats increased caspase-3 expression in the following manner TME> DOX. Figure 5F also shows that, the combined administration of TME with DOX improved the apoptotic effect of DOX. Fig. (5G) depicts the area % of the immunohistochemical staining of the expression levels of caspase-3 in liver sections.



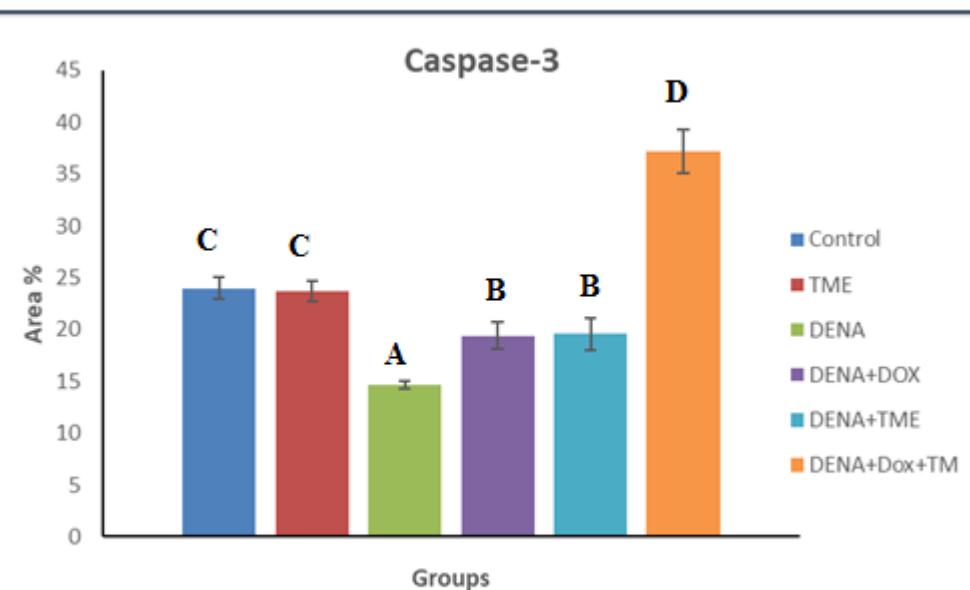


Fig. 5(G): The mean of area % of caspase-3 in control groups and under the influence of different treatments
Values are mean \pm SE (n= 6). The presence of different letter on each bar means a significant difference between groups. ANOVA test followed by Duncan's multiple comparisons between groups at P< 0.05 were employed

IV. Discussion

The present work was conducted for the first time to evaluate the curative role of *Terminalia muelleri* (TM) in the form of ethanolic leaf extract against DENA-induced HCC. In addition to evaluate its role as a combined therapy with DOX which is one of the standard chemotherapy. Diethyl nitrosamine (DENA) is one of the most potent hepatocarcinogenic agent that is mostly used to induce lesions in rats that mimic different kinds of benign and malignant tumors in human.^[18,1] The administration of DENA for six weeks resulted in a significant elevation in the activity of ALT and AST. The leakage of these enzymes in the circulation indicated the incidence of damaged liver cells.^[27,28,1] Moreover, the microscopic examination of DENA-challenged liver revealed an obvious cellular damage with changes in liver architecture with well differentiated HCC accompanied by dilatations of portal vein with multiple newly formed bile ductules, oedema and few inflammatory cells infiltration surrounding by hepatocyte. Also, DENA induced polarity and pleomorphism of the anaplastic hepatocytes, karyomegalocytes with prominent nucleoli of the anaplastic hepatocyte. In the present study, the induction of HCC is assured by the significant increase in SAFP after six weeks of DENA injection. This finding is in the same line with that of Cui *et al.*^[29] who reported that, the overexpression of AFP has been associated with uncontrolled growth of HCC.

In the present study, the development and the progression of HCC is manifested by the significant and progressive increase in SAFP level of DENA-induced HCC rats at the end of the experimental period (14 weeks). Data of the present study revealed that, the induction and progression of HCC by DENA could be also manifested by the significant decrease in serum Albumin (SALB) level (Table 2). Our results were in the harmony with the results of Elsadek *et al.*^[30], Elguindy *et al.*^[31], Carr and Guerra.^[32] This reduction in albumin may reflect the decrease of hepatic capacity to synthesize protein that could be accounted to cell necrosis.^[33] Moreover, the obtained decrease in SALB level after the induction of HCC is negatively correlated with the obtained increase in SAFP level, suggesting that serum ALB level has the significance in the manifestation of HCC and confirmed the finding of Carr and Guerra^[32] who reported that, HCC patient with lower serum ALB had significantly large maximum tumor diameter, greater prevalence of portal vein thrombosis and higher AFP level.

The group of rats treated with TME displayed a well-marked decrease in SAFP level compared not only to DENA-induced HCC rats but also with DOX treated rats that reflected the superior therapeutic activity of *T. muelleri* and proved the efficacy of the examined extract in inhibiting the progression of tumor cells. The

obtained data also revealed that, the co-administration of DOX with TME exerted more pronounced effect in decreasing the serum AFP.

Proliferating cell nuclear antigen (PCNA) represents an important cellular marker for assessing the proliferation during hepatocarcinogenesis.^[34] The positive expression of PCNA is considered as a common index for hepatocyte proliferation due to PCNA functions as a cofactor of DNA polymerase and that it is directly involved in DNA replication.^[35] Therefore, PCNA represents an important cellular marker for assessing the proliferation during hepatocarcinogenesis. In the current study, the expression of PCNA was examined immunohistochemically in livers of the examined groups. The elevated expression of PCNA in the liver sections of DENA-treated animals indicated the accelerated cell proliferation during an early phase of rat liver tumorigenesis, which supported the finding of **Bishayee and Dhir** ^[36]and **Bishayee et al.** ^[37]

The exploration of agents that can affect abnormal proliferation of hepatocytes may be of great value in the prevention of HCC.^[38] Data of the present study revealed a significant decrease in the immunohistochemical staining expression of PCNA due to TME and DOX administration. This finding proved the antiproliferative mechanisms of *T. muelleri* phytochemicals that fundamentally contributed to the inhibition of DENA-induced tumorigenesis. The results of current study are in consistent with several studies confirmed the antiproliferative effects of various phytochemicals compounds in several cancer models.^[39,10] and proved the anti-proliferative effect of TME is able to either block or retard entire steps of carcinogenesis

In the present study, caspase-3 was immunohistochemically detected to identify cells undergoing apoptosis. The obtained data revealed a significant decrease in the expression level of caspase-3 in DENA treated challenged rats. This finding is in consistent with the study of **Zhang et al.** ^[40], **Akshatha et al.** ^[41], **Singh et al.** ^[13]. Moreover, **Wang and Wang**^[42] demonstrated that, the role of AFP is not only in the proliferation of HCC cells with creation of tumor blood vessels, but as well augments the antiapoptotic effect of cancer cells, this finding is clearly proved in the current study through the obtained increase in AFP level in association with significant decrease in Caspase – 3 expression level.

Immunoreactivity of caspase-3 toward hepatocytes in the liver sections of the group of rats treated with the standard chemotherapy drug, DOX, was analyzed to compare the efficacy of the target herbal medicine. The results of the current study revealed that, the expression level of caspase-3 reduced in the DENA- induced HCC group and was significantly elevated after TME and DOX administration and this effect was more pronounced in the TME group than the DOX-treated one. This finding proved that caspase-3 expression plays an imperative role in the development of the HCC condition and TME can act predominantly through the overexpression of caspase-3.

Histological investigations of liver sections provided an additional strong evidence for the combined TME and DOX beneficial effects to combat DENA-induced HCC. In the current study, the architecture of liver tissue in DENA-exposed rats was highly altered as evidenced by the appearance of multiple degenerated cells, loss of architecture and tumoral vacuoles in H and E staining (Fig. 3C). These findings are in consistent with the results of **Gupta et al** ^[9], **Elguindy et al.**^[31]. This may indicate that DENA is principally metabolized in liver and the reactive metabolites produced are responsible for degenerating hepatocytes.

The individual administration of either TME or DOX failed to totally compensate the DENA induced the incidence of histopathological alterations. However, the combined administration of TME and DOX showed pronounced improvement in the hepatocytes disorganization and degeneration when compared to the DENA-induced rats. This enhancement may be mainly contributed to TME administration that can effectively decrease reactive metabolites of DENA inside hepatocytes by avoiding oxidative tension caused cellular damage.^[43, 44] In the current study, the obtained findings reflect the effective role of TME polyphenols in modulating HCC progression. The obtained findings in the present study are in the same line with and support the recorded findings of **Murugan et al.** ^[45], **Liu et al.** ^[46]and **Wang et al.** ^[47] who provided evidence that polyphenols have potentials chemopreventive and chemotherapeutic effects.

V. Conclusion

In conclusion TME could be a promising or adjuvant therapy in the treatment of HCC.

Conflicts of interest

The author declares no conflict of interest.

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