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Synergistic Protective Effects of The Nanoemulsion of Thyme and Basil Essential Oils Against the Oxidative Damage, Cytotoxicity, and DNA Fragmentation of Titanium Dioxide Nanoparticles in Rats

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Abstract

This study aimed to compare the protective role a crude mixture of thyme and basil essential oils (TO and BO) and their nanoemulsion (TON and BON) against oxidative damage, cytotoxicity and DNA fragmentation in rats exposed to titanium dioxide nanoparticles (TiO_2-NPs). Six groups of male Sprague-Dawley rats were subjected to oral treatment for 3 weeks including, the control group, the group treated with TO plus BO (5 mg/kg b.w each), the group treated with TON plus BON (2.5 mg/kg b.w each), the group treated with TiO_2-NPs (50 mg/kg b.w) and the groups treated with TiO_2-NPs plus the TO and BO or TON plus BON. Blood and tissue samples were collected for different bioassays, cytogenetic and histological examination. The results revealed that TiO_2-NPs disturbed the liver and kidney indices, lipid profile, serum cytokines oxidant/antioxidant markers, antioxidant gene expression and histological structure in the liver. Both TO plus BO or TON plus BON could prevent these disturbances and TON plus BON were more effective. These outcomes indicated the synergistic effect of the oils and the role of nanoemulsion in the enhancement of the antioxidant efficiency beside expand their dispersibility and foster their controlled delivery in addition to the antioxidant property of WPI. It could be concluded that TON and BON are safe and can be applied for the preparation of eco-friendly antioxidant for food and medical proposes.

Keywords**:** thyme essential oil; basil essential oil; nanoemulsion; titanium dioxide nanoparticles; oxidative damage; antioxidant

Introduction

Essential oils (EOs) are a complex mixture of volatile organic compounds extracted from various aromatic and medicinal herbal plants as secondary metabolites to protect the plants from microorganisms [1,2]. Therefore, the FDA (Food and Drug Administration) recognized them as safe compounds. Its chemical composition can vary between species of aromatic plants and varieties and within the same of geographical areas [3]. Various methods to separate them include solvent extraction, steam distillation, cold press extraction, maceration, water distillation, and CO_2 extraction [4]. Nowadays, EOs are used for the treatment of health problems due to their pharmacological activity and several biological activities such as suppressing the free radicals generation, anticancer, antioxidant, antifungal, antiviral, antibacterial, anti-inflammatory, antimutagenic, antidiabetic, and immunomodulatory properties [5-9].

Thyme essential oil (TEO) exhibited various beneficial biological activities, including antitumor, antioxidant, antimicrobial, and anti-inflammatory properties [10,11]. Basil EO (BEO) is rich in

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terpenoid and phenolic components [12]. This oil has different biological activities such as inhibit cholesterol synthesis and enhance digestion [13], anti-inflammatory [14], antimicrobial [15,16], antioxidant [17,18], chemopreventive agents [19], and anthelmintic [20]. The sensory attributes of EOs are very important when these EOs are utilized in food or pharmaceutical applications [21]. Hence, the organoleptic properties of EOs should be considered when high levels of EOs are required to obtain an effective antioxidant effect [22]. To overcome these limitations in the application of EOs, a combination of lower concentrations can be used to decrease the adverse organoleptic effects and achieve maximum bioactivity [23, 24].

Moreover, the low solubility and stability, together with the high volatility of these EOS in biological fluids, is an obstacle to their absorption and leads to very low bioavailability. Consequently, these factors limit the utilization of EOs as candidates for pharmaceuticals and treatments. A promising technique to overcome these problems is the encapsulation technology which can increase the physical stability of different bioactive compounds, protect the EOs from the environmental conditions, modulate the EOs release, reduces their volatility, and enhances their bioactivity [25-28].

Titanium dioxide nanoparticles (TiO_2-NPs) are white crystal powder with sizes less than 100 nm and have a high refractive index, absorptive qualities, anticorrosive and photocatalytic properties [29,30]. Owing to these properties, these particles are widely used in various industrial and consumer products, including nutritional supplements, production of toothpaste, skincare products, paints production, and food colorants [31,32]. Additionally, they are utilized as nano-drug in the remedy of some diseases [33], and as nanocarriers for drug delivery [34]. The International Agency for Research on Cancer (IARC) classified the TiO_2-NPs as group 2B (probably carcinogenic to humans) [35]. Exposure to TiO_{γ}-NPs occurs through injection, inhalation, oral ingestion, and skin absorption [36] because of its physicochemical characteristics like its small size, high surface area, and high reactivity. Several reports postulated many cellular impacts of NPs, including the induction of oxidative stress, inflammatory reactions, DNA damage, and cell apoptosis [33,37,38]. Therefore, this work was performed to show the synergistic pharmaceutical effects of TON and BON against TiO_2-NPs-induced oxidative damage, disturbances in gene expression, and DNA damage in rats.

Materials and methods

Materials

Thyme oil (TO) and basil oil (BO) were provided by the oil extraction unit, National Research Centre, Dokki, Cairo, Egypt. They were analyzed by the GC-MS, which identified 17 bioactive compounds in the TO and 55 compounds in BO. The major compounds in TO were thymol and carvacrol (64.24% and 22.34 %, respectively); however, linalool and methyl chavicol were the major compounds in BO (53.9% and 12.63%, respectively). Whey protein isolate (WPI) was supplied by Sigma-Aldrich (St. Louis, MO, USA). Titanium dioxide nanoparticles (TiO_2-NPs) were biosynthesized in our laboratory as described in our previous work [39] using the extract of orange peel (EOP), and the particle size was 50 ± 2.4 nm and the zeta potential was -30.44 mV

Nanoencapsulation of EOs (TON and BON)

As described previously, TON and BON were prepared using WPI as a wall [37]. Tween 80 was used as an emulsifier at a ratio of 2:1 w/w according to Jinapong et al. [40] then the emulsion was encapsulated by spray drying. TON particles were irregular in shape with an average size of 230 ± 3.7 nm and a ζ-potential of -24.17. However, the particles of BON were semi-rounded in shape with an average size of 120 ± 4.5 nm and a ζ-potential of -28 ± 1.3 mV.

Chemicals and kits

The chemicals and kits used in this study and their sources are displayed in Table (1).

Experimental animals

Male Sprague-Dawley rats (3 months old, 155 ± 15 g) were supplied by the Experimental Animal Facility, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt.

TABLE 1. Chemicals and kits

Name of chemical and kits	Source
Alanine transaminase (ALT)	Randox Co. (Antrim, UK).
Aspartate transaminase (AST)	
Creatinine	
Urea	
Cholesterol (Cho)	FAR Diagnostics Co. (Via Fermi, Italy).
Triglycerides (TriG)	
High-density lipoprotein (HDL)	
Low-density lipoprotein (LDL)	

Albumin (Alb)	
Total protein (TP)	
Malondialdehyde (MDA)	Oxis Research TM Co. (USA).
Nitric oxide (NO)	Eagle diagnostics (Dallas, TX, USA).
Glutathione peroxidase (GPx)	
Superoxide dismutase (SOD	
Catalase (CAT)	
Alpha-fetoprotein (AFP)	BiochemImmuno Systems Co. (Montreal, Canada)
Carcinoembryonic antigen (CEA)	Invitrogen (Germany)
Tumor necrosis factor-alpha (TNF-α)	
TRIzol® reagent	
RNAse-free DNAse	
First Strand cDNA Synthesis	iNtRON Biotechnology (Seoul, Korea)
SYBR® Premix Ex TaqTM	TaKaRa Biotech. Co. Ltd. (Shiga, Japan)

The rats were housed in cages in a ventilated animal room (12 h dark/ light cycle, 25 ± 1°C, and 25-30% humidity) at the Animal House Lab, Faculty of Pharmacy, Cairo University, Cairo, Egypt, and were given free access to water and rodent show diet. All animals were adapted for 1 week before the starting of the experiment and all the procedures used in this experiment complied with the guidelines of the National Institute of Health (NIH publication 86-23 revised 1985). The Research Ethics Committee Research Ethics Committee approved the protocol approved the protocol of Faculty of Pharmacy, Cairo University (REC-FOFCU), Cairo, Egypt.

Experimental design

Animals were distinguished randomly into 6 groups (10 rats/group) and treated orally for 3 weeks as follows: group 1, the untreated control group; group 2, animals that were treated with TO plus BO (5 mg/kg b.w each); group 3, animals that treated with TEON plus BEON (2.5 mg/kg b.w each); group 4, animals that treated with TiO_2-NPs (50 mg/kg b.w); group 5, animals that treated with TiO_2-NPs plus TO and BO, and group 6, animals that treated with TiO_2-NPs plus TEON and BEON. After the termination of the experimental period (i.e. day 21), animals were fasted for 12 h, and then blood samples were collected under isoflurane anesthesia through the retro-orbital venous plexus. The sera were collected by centrifuging at 3000 rpm under cooling (4°C) for 10 min and then kept at -20 °C even used for the determination of liver and kidney parameters, lipid profile, and serum cytokines (AFP, TNF-α, and CEA) according to the kit's instructions. Afterward, the animals were euthanized, then samples of liver and kidney of each animal were collected, weighed, homogenized in phosphate buffer (pH 7.4), and centrifuged at 1700 rpm, and 4 ºC for 10 min. The supernatant was used to estimate the SOD, CAT, GPx, MDA, and NO [41]. Another liver sample of each animal was quickly frozen using liquid nitrogen and kept at -80 ºC for genetic analysis. Additionally, each animal's sample of the liver tissue was fixed in 10% formal saline for histopathological examination under a light microscope [42].

Gene expression analysis

Isolation of total RNA

TRIzol®reagent was utilized to isolate the total genomic RNA from frozen liver tissues (- 80 ◦C) of all treated animals following the manufacturer's protocol. The concentration and purity of RNA were determined by NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA). The RNA pellets were stored in DEPC treated water then treated with the RNAse-free DNAse kit to digest the potential DNA residues and the RNA aliquots were kept at -20 °C until use for the reverse transcription

DNA fragmentation assay

Real-Time-PCR analysis **(**RT-qPCR)

SYBR® Premix Ex TaqTM kit was used to assay the qRT-PCR analyses as previously described [43]. The gene-specific primer sequences for GAPDH, GPx, SOD, and CAT are presented in Table (2). RRT-qPCR was carried out on Stratagene Mx3005P Real-Time PCR System (Agilent Technologies). Relative gene expression levels were normalized to GAPDH. RT-qPCR data were obtained as a threshold cycle (Ct) value and were used to calculate ΔCt values for each sample [44,45].

The $2^{-\Delta\Delta CT}$ method was applied for the determination of the quantitative values of the specific genes to (GAPDH), and the relative quantification of the target gene to the reference was calculated using the following $2^{-\Delta\Delta CT}$ equations:

ΔCT(test) = CT(target, test) − T(reference, test),

ΔCT (calibrator) = CT(target, calibrator) − CT(reference, calibrator),

$$\Delta\Delta CT = \Delta CT(Test) - \Delta CT(calibrator)$$

DNA was isolated from hepatic tissue following Kuo et al. [46] and the colorimetric estimation of DNA content was detected as described by Perandones et al. [47]. Both supernatant and the

pellet were used for DPA assay after acid extraction of DNA. The following formula was used to calculate the percentage of DNA fragmentation:

% DNA fragmentation = (O.D. of supernatant /O.D. of supernatant + O.D. of the pellet) × 100.

Statistical analysis

The data were statistically analyzed by computerized software SPSS (Statistical Package of Social Science, version 20, Armonk, New York: IBM Corp). One-way analysis of variance (ANOVA) followed by Duncan's multiple comparisons test to determine the difference among the experimental groups. The level of statistical significance was set at $P < 0.05$.

TABLE 2. Details giving primer sequences for the genes amplified

cDNA	Accession number	Forward primer	Reverse primer	RT-PCR product size (pb)	Reference
GAPDH	NM_017008.4	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG	496 pb	[121]
Cu-Zn SOD	FQ210282.1	GCAGAAGGCAAGCGGTGAAC	TAGCAGGACAGCAGATGAGT	477pb	[122]
GPx	NM_030826.4	CTCTCCGCGGTGGCACAGT	CCACCACCGGGTCGGACATAC	290 pb	[122]
CAT	NM_012520.2	GCGAATGGAGAGGCAGTGTAC	GAGTGACGTTGTCTTCATTAGCACTG	652 pb	[123]

Results

The results showed that TiO_2-NPs administration elevated the serum AST, ALT, D.BIL, T.BIL, urea, creatinine, and uric acid and a significantly decreased serum Alb and TP (Table 3). Animals that were administrated TO plus BO showed a marked decrease in D.BIL, urea, creatinine, and uric acid compared to the control; however, the other parameters were in the normal range of the control. The group that received TON plus BON showed a notable decrease in T.BIL, D.BIL, urea, and creatinine and a marked increase in uric acid; however, the other parameters were in the normal range of the control group. The animals that administrated TiO_2-NPs plus TO and BO exhibited a marked decrease in AST, D.BIL, urea, creatinine, and uric acid compared with those that received TiO_2-NPs alone accompanied by a significant increase in Alb and normalized ALT, TP, and T.BIL. However, animals that administrated TiO_2-NPs plus TON and BON were similar to the control in AST, ALT, Alb, T.BIL, and creatinine and showed a marked improvement in TP, D.BIL, uric acid, and urea.

Treatment with TiO_2-NPs showed significant disturbances in the lipid profile (Table 4), since Cho, TriG, and LDL-Cho were markedly elevated, and HDL-Cho was reduced significantly compared to the control. Treatment with TO plus BO or TON plus BON induced a significant decrease in LDL-Co but did not affect the other lipid parameters. However, treatment with TiO_2-NPs plus TON and BON caused a considerable improvement in the lipid profile parameters compared to TiO_2-NPs-alone-treated animals. They restored Cho to the normal level of the control. Meanwhile, administration of TiO_2-NPs plus TON and BON could normalize TriG, HDL-Cho, and LDL-Cho and decreased Cho than the control level.

The data depicted in Table (5) showed the results of serum cytokines. These data revealed that administration of TiO_2-NPs increased AFP, TNFα, and CEA; however, TO plus BO or TON plus BON induced a marked reduction in the serum cytokines, and TON plus BON was more effective than TO plus BO. The animals that received TiO_2-NPs plus TO plus BO or TON plus BON showed a significant improvement in serum cytokines compared to the group that received TiO_2-NPs alone, and TON plus BON was more effective than TO plus BO.

TABLE 3. Effects of TO plus BO and TON plus BON on serum biochemical parameters in rats treated with TiO_2-NPs

Groups Parameter	Control	TO + BO	TON + BON	TiO_2-NPs	TiO_2-NPs + TO + BO	TiO_2-NPs + TON + BON
AST (U/L)	148.01 ± 1.3٩^a	145.38 ± 5.65^a	1٤8.45 ± 6.20^a	205.81 ± 1.88^b	161.08 ± 1.18^c	145.86 ± 1.86^a
ALT (U/L)	45.11 ± 1.0٧^a	46.08 ± 2.63^a	46.12 ± 2.35^a	85.91 ± 1.32^b	48.11 ± 3.38^a	44.98 ± 3.37^a
Alb (mg/dl)	2.89 ± 0.03^a	2.62 ± 0.02^a	2.71 ± 0.06^a	1.07 ± 0.02^b	2.25 ± 0.05^c	2.92 ± 0.08^a
TP (g/dl)	6.39 ± 0.18^a	6.33 ± 0.35^a	6.37 ± 0.29^a	4.19 ± 0.10^b	6.00 ± 0.14^a	7.07 ± 0.07^c
T.BIL (mg/dl)	0.9١ ± 0.02^a	0.90 ± 0.01^a	0.84 ± 0.01^b	1.٨٠ ± 0.10^c	0.92 ± 0.03^a	0.82 ± 0.01^a
D.BIL (mg/dl)	0.19 ± 0.02^a	0.05 ± 0.01^b	0.07 ± 0.01^c	0.4١ ± 0.01^d	0.30 ± 0.01^e	0.22 ± 0.01^f
Creatinine (mg/dl)	0.93 ± 0.02^a	0.73 ± 0.04^b	0.64 ± 0.03^c	2.6٥ ± 0.10^d	2.02 ± 0.01^e	0.95 ± 0.02^a
Urea (mg/dl)	54.97 ± 0.53^a	47.94 ± 2.70^b	46.24 ± 3.79^b	81.28 ± 0.55^c	62.57 ± 0.67^d	57.34 ± 1.12^e
Uric acid (mg/dl)	1.٤٢ ± 0.03^a	1.22 ± 0.14^b	1.57 ± 0.13^c	3.15 ± 0.04^d	2.33 ± 0.18^e	1.59 ± 0.03^c

Within each row, means superscripts with different letters are significantly different ($P < 0.05$)

TABLE 4. Effects of TO plus BO and TON plus BON on lipid profile parameters in rats treated with TiO_2-NPs

Groups parameter	Control	TO + BO	TON + BON	TiO^2-NPs	TiO_2-NPs + TO + BO	TiO_2NPs + TON + BON
Cho (mg/dl)	65.87 ± 0.86[a]	63.88 ± 4.52[a]	63.06 ± 2.21[a]	92.61 ± 1.31[b]	66.87 ± 1.62[a]	59.92 ± 0.7٢[c]
Tri G (mg/dl)	83.92 ± 1.61[a]	83.12 ± 3.97[a]	83.30 ± 1.21[a]	120.73 ± 0.72[b]	104.71 ± 2.17[c]	81.90 ± 0.77[a]
HDL-Cho (mg/dl)	33.32 ± 1.31[a]	34.66 ± 1.00[a]	35.70 ± 2.10[a]	19.93 ± 0.38[b]	30.67 ± 0.39[c]	30.99 ± 0.47[a]
LDL-Cho (mg/dl)	34.68 ± 0.44[a]	31.97 ± 0.89[b]	31.02 ± 2.91[b]	63.65 ± 0.97[c]	38.78 ± 0.87[d]	33.85 ± 0.46[a]

Within each row, means superscripts with different letters are significantly different (P< 0.05)

TABLE 5. Effects of TO plus BO and TON plus BON on serum cytokines in rats treated withTiO_2-NPs

Groups parameter	Control	TO + BO	TON + BON	TiO^2-NPs	TiO_2-NPs + TO + BO	TiO_2NPs + TON + BON
AFP (ng/ml)	0.053 ± 0.002[a]	0.031 ± 0.01[b]	0.034 ± 0.01[b]	0.22 ± 0.007[c]	0.11 ± 0.01[d]	0.06 ± 0.01[e]
TNF-α (ng/ml)	0.45 ± 0.01[a]	0.26 ± 0.02[b]	0.25 ± 0.02[b]	0.69 ± 0.01[c]	0.71 ± 0.02[d]	0.50 ± 0.02[e]
CEA (ng/ml)	3.17 ± 0.09[a]	1.57 ± 0.15[b]	1.51 ± 0.22[b]	4.01 ± 0.14[c]	2.50 ± 0.04[d]	2.19 ± 0.02[e]

Within each column, means superscripts with different letters are significantly different (P < 0)

The results of antioxidant enzymes in the liver and kidney are presented in Table (6). TiO_2-NPs administration decreased the hepatic and renal CAT, SOD, and GPx than the control group. Animals that received TO plus BO increased the hepatic SOD and CAT, also the renal SOD and GPx, but did not influence the hepatic GPx and the renal CAT. However, administration of TON plus BON increased the hepatic CAT, SOD, and the renal GPx but did not influence the hepatic GPx or the renal SOD and CAT. Co-administration of TiO_2-NPs plus TO and BO significantly improved the antioxidant enzyme activity in both liver and kidney compared to the animals that received TiO_2-NPs alone. Furthermore, the co-administration of TiO_2-NPs plus TON and BON restored all the antioxidant enzyme activity in the liver and kidney except SOD which was still lower than the control.

The data on MDA and NO in both organs (Table 7) revealed that TiO_2-NPs increased MDA and NO in both tissues. Administration of TO plus BO or TON plus BON did not display any marked effects on MDA and NO in both organs except the renal NO, which still decreased than the control group. The animals administrated TiO_2-NPs plus TO, and BO or TON and BON showed a marked improvement in MDA and NO in both organs, and TON plus BON was more effective than TO plus BO.

Quantitative analysis of hepatic mRNA expression

The results of mRNA expression of the hepatic antioxidant enzymes, GPx (Fig. 1A), SOD (Fig. 1B), and CAT (Fig. 1C) indicated that TiO_2-NPs down-regulated the expression of these genes compared with the control animals. Treatment with TO plus BO or TON plus BON induced a significant up-regulation of the mRNA expression levels of the target genes, and TON plus BON was more effective. Co-administration with TiO_2-NPs plus the TO plus BO or TON plus BON improved the mRNA expression of the tested genes toward the control level although none of these agents could normalize the mRNA expression of these genes; meanwhile, no notable difference was observed between the animals that received TiO_2-NPs plus TO and BO or the group received TON and BON.

TABLE 6. Effects of TO plus BO and TON plus BON on hepatic and renal oxidative stress markers in rats treated with Tio_2-NPs

parameters Groups	Liver			Kidney		
	CAT (mU /g)	GPx (U/mg)	SOD (U/mg)	CAT (mU/g)	GPx (U/mg)	SOD (U/g)
Control	6.69 ± 0.08[a]	37.18 ± 1.09[a]	28.70 ±0.66[a]	8.74 ± 0.15[a]	31.93 ±0.99[a]	28.75 ± 0.50[a]
TO + BO	10.87 ± 0.42[b]	38.20 ± 0.71[a]	32.94 ± 1.47[b]	9.64 ± 0.43[a]	35.04 ± 1.77[b]	34.02 ± 0.73[b]
TON + BON	11.55 ± 0.54[c]	38.88 ± 0.68[a]	34.49 ± 1.24[c]	9.98 ± 0.44[a]	36.42 ± 2.5[d]	33.90 ± 1.31[a]
TiO_2-NPs	2.93 ± 0.15[d]	21.86 ± 0.56[b]	14.54±0.15[d]	3.53 ± 0.15[b]	12.26 ± 0.58[e]	15.02 ± 0.25[c]
TiO_2-NPs + TO + BO	4.95 ± 0.06[e]	32.65 ± 0.68[c]	23.48 ± 0.48[e]	٦.95 ± 0.13[c]	24.83 ± 0.69[f]	22.23 ± 0.50[d]
TiO_2-NPs + TON + BON	6.96 ± 0.23[a]	36.62 ± 0.61[a]	24.06 ± 0.56[e]	9.2٧ ± 0.٢١[a]	30.78 ± 0.80[a]	26.11 ± 1.14[e]

Within each column, means superscripts with different letters are significantly different (P < 0)

TABLE 7. Effects of TO plus BO and TON plus BON on hepatic and renal oxidative stress markers in rats treated with Tio_2-NPs

parameters / Groups	Liver		Kidney	
	MDA (nmol/g)	NO (µmol/g)	MDA (nmol/g)	NO (µmol/g)
Control	139.30 ± 1.61[a]	132.48 ± 0.58[a]	185.20 ± 1.42[a]	137.66 ± 4.47[a]
TO + BO	137.96 ± 2.73[a]	130.54 ± 0.06[a]	182.24 ± 5.57[a]	118.9 ± 0.08[b]
TON + BON	135.56 ± 7.47[a]	127.08 ± 0.08[a]	185.62 ± 3.99[a]	120.91 ± 0.21[b]
TiO_2-NPs	213.80 ± 3.82[b]	321.88 ± 6.91[c]	270.24 ± 2.15[c]	348.74 ± 3.79[c]
TiO_2-NPs + TO + BO	175.60 ± 4.23[c]	165.6 ± 2.19[d]	234.63 ± 1.87[d]	287.81 ± 2.42[d]
TiO_2NPs + TON + BON	152.22 ± 1.63[d]	158.04 ± 2.72[f]	191.86 ± 3.15[e]	216.7 ± 3.37[e]

Within each column, means superscripts with different letters are significantly different ($P < 0.05$)

Colorimetric DNA fragmentation

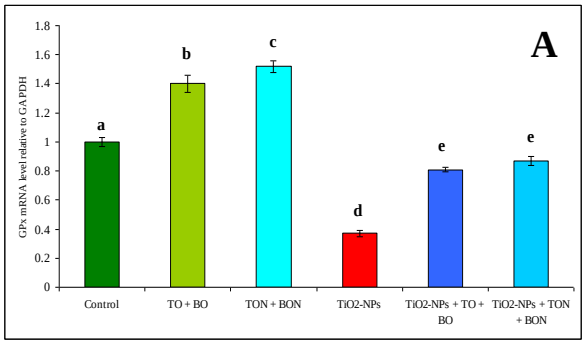
The current data also revealed that the hepatic DNA fragmentation percent in TiO2-NPs-treated rats was significantly increased ($P \leq 0.05$) than in the negative control animals (Table 8). Treatment with TO plus BO or TON plus BON significantly decreased the DNA fragmentation percentage. However, co-administration of TiO_2-NPs plus TO plus BO or TON plus BON reduced the percentage of DNA fragmentation compared to the TiO_2-NPs alone-treated group, and the change in DNA fragmentation reached 1.1 and 0.76% in the animals that received either TiO_2-NPs plus TO and BO or TiO_2-NPs plus TON and BON, respectively. The inhibition in DNA fragmentation percent reached 58.88.3% in TO plus BO-treated group, while the inhibition retched 62.1% in the TON plus BON-treated group. Additionally, the agarose gel electrophoresis of the DNA (Fig. 2) confirmed the colorimetric assays of DNA fragmentation, the antioxidant status and corroborated the changed levels of the gene transcripts. The liver samples of TiO_2-NPs-intoxicated rats showed a smear (a hallmark of necrosis), DNA fragmentation with ladder formation, indicating random DNA degradation (Fig. 2, Lanes 2) when compared with the control (Fig. 2, Lanes 1), and either TO plus BO or TON plus BON-treated (Fig. 2, Lanes 3 and 4) groups. Treatment of TiO_2-NPs -intoxicated rats with either TO plus BO or TON plus BON markedly suppressed DNA fragmentation (Fig.2, Lanes 5,6), where DNA was still localized at the starting point. No significant difference existed between the DNA electrophoretic patterns of either TO plus BO or TON plus BON-treated rats and the control groups.

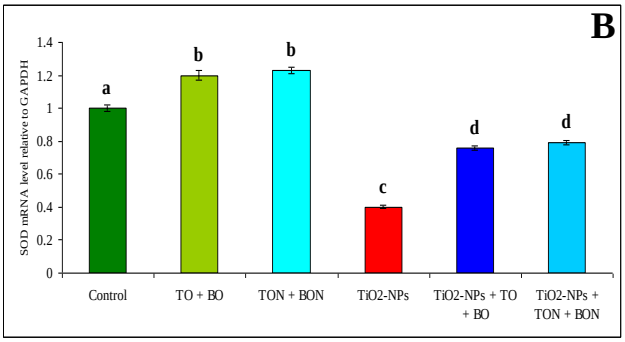
Histological results

The examination of the sections in the control liver showed the normal central vein, hepatic lobule structure, and blood sinusoid (Fig. 3A). The liver sections of the rats that received TO plus BO showed normal liver architecture, slight dilatation of blood sinusoid, and few inflammatory cells (Fig. 3B). The examination of rat liver that received TON plus BON showed a relatively normal histological pattern, aggregation of inflammatory cells, some nuclei are pyknotic, and peripheral chromatin clumping (Fig. 3C). However, the sections of the rats' liver that received TiO_2-NPs showed dilatation in the portal tract, proliferation in the bile duct epithelial cells, and the hepatocytes revealed vacuolar degeneration, and nuclei appeared small in size. At the same time, some of them were pyknotic, necrotic, karyolysis, and peripheral chromatin clumping were observed (Fig. 3D). The liver of rats that received TiO_2-NPs plus TO and BO showed some improvements, the hepatic tissue relatively normal around the central area, and slight vacuolar degeneration, while the nuclear damage in the form of binucleation, pyknosis, karyolysis were still presented (Fig. 3E). The liver sections of the rats that received TiO_2-NPs plus TON and BON showed more improvements representing marked diminution of fibrosis, the structure of hepatic tissue almost normal around the portal area, and slight diffusion of inflammatory cells (Fig. 3F).

TABLE 8. DNA fragmentation in the hepatocytes of rat treated with TO + BO or TON + BON alone or in combination with TiO_2-NPs

Treatment	DNA fragmentation%	
	Mean (% ± S.E.)	DNA fragmentation inhibition %
Control	3.3 ± 0.03[b]	
TO + BO	2.7 ± 0.08[a]	
TON + BON	2.5 ± 0.07[a]	
TiO_2-NPs	10.7 ± 0.25[e]	
TiO_2-NPs + TO+BO	4.4 ± 0.17[c]	58.88
TiO_2NPs + TON + BON	4.06 ± 0.06[d]	62.1





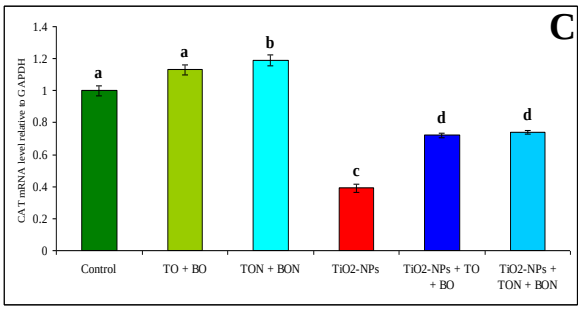


Fig.1. Effect of TO + BO or TON + BON on relative expression of (A) GPx, (B) SOD and (C) CAT gene in liver of rats with treated with TiO_2-NPs.
Analyses were performed in triplicate. Data are the mean ± SE of three different liver samples in same group. Columns carrying different superscript letters are significantly different at $P \leq 0.05$.

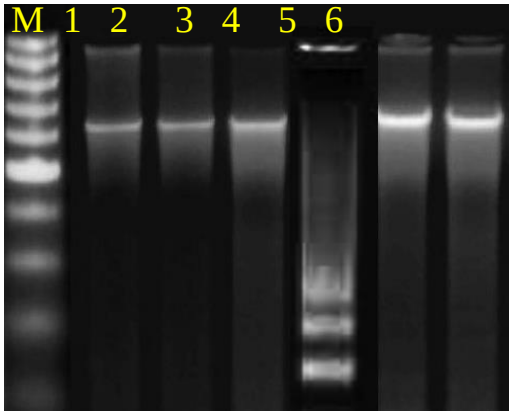


Fig. 2. Agarose gel electrophoresis of DNA extracted from liver of rats in different treatment groups showing the DNA fragmentation. Lane M, 100 bp DNA ladder; Lane 1 untreated control group; Lane 2, TO + BO -treated group; Lane 3, TON + BON-treated group, lane 4, TiO_2-NPs; Lane 5 TO + BO + TiO_2-NPs-treated group and lane 6 TON + BON +TiO_2-NPs -treated group.

Histological results

The examination of the sections in control liver showed the normal central vein, hepatic lobule structure, and blood sinusoid (Fig. 3A). The liver sections of the rats that received TO plus BO showed normal liver architecture, slight dilatation of blood sinusoid, and few inflammatory cells (Fig. 3B). The examination of rat liver that received TON plus BON showed a relatively normal histological pattern, also, aggregation of inflammatory cells, some nuclei are pyknotic and peripheral chromatin clumping (Fig. 3C). However, the sections of the rats liver that received TiO_2-NPs showed dilatation in the portal tract, proliferation in the bile duct epithelial cells, and the hepatocytes revealed vacuolar degeneration, nuclei appear small in size while some of them were pyknotic, necrotic, karyolysis and peripheral chromatin clumping were observed (Fig. 3D). The liver of rats that received TiO_2-NPs plus TO and BO showed some improvements, the hepatic tissue relatively normal around the central area, slight vacuolar degeneration, while the nuclear damage in the form of binucleation, pyknosis, karyolysis was still presented (Fig. 3E). The liver sections of the rats that received TiO_2-NPs plus TON and BON showed more improvements representing marked diminution of fibrosis, the structure of hepatic tissue almost normal around the portal area, and slight diffusion of inflammatory cells (Fig. 3F).

Discussion

In this work, we evaluated the possible protective role of TON and BON against the TiO_2-NPs-induced oxidative damage compared to the crude TO and BO. The doses of TiO_2-NPs, TON, and BON were selected based on our previous works [٤٥,48,49], respectively. The data revealed that TiO_2-NPs induced severe disturbances to different organs, especially the liver and kidney, after the absorption by the GIT. These results were compatible with what was mentioned in the scientific literature [50,51]. Treatment with TiO_2-NPs showed severe disturbances in the biochemical parameters related to liver and kidney functions. Significant elevations in serum AST, ALT, urea, creatinine, uric acid, total BIL, direct BIL, hepatic and renal NO, and MDA accompanied by a significant reduction in Alb, TP,

hepatic and renal GPx, SOD, and CAT were remarked in this group. Also, the elevation in serum TriG, Cho, and LDL-Cho and the reduction in HDL-Cho and the histological alteration in the hepatic tissue. These outcomes suggested that both the liver and kidney are the target organs of TiO_2-NPs toxicity. These particles change their function resulting in a state of oxidative damage similar to those reported previously [29,52-55]. The increase in liver enzymes suggests the hepatocytes' injury or damage and the release of these enzymes to the extracellular fluids [56]; however, the elevation of kidney parameters revealed the damage or dysfunction of the kidney [57]. In addition, the disturbances in lipid profile indicate that TiO_2-NPs affect the function of lipoprotein lipase enzyme or the ability to remove or transform the lipid fractions [58,59]. The reduction of HDL-Cho and the elevation of Cho, TriG, and LDL-Cho also postulated that exposure to TiO_2-NPs may be a causative risk factor for cardiovascular diseases [54,60,61].

Moreover, the elevated inflammatory cytokines (TNF-α, AFP, and CEA), hepatic and renal NO, and MDA with the inhibition of antioxidant enzyme activity (GPx, SOD, and CAT) postulated that oxidative stress is the prime mechanism of TiO_2-NPs toxicity. These results also indicated that TiO_2-NPs disturb the redox balance and stimulate ROS formation, mainly hydroxyl radical (•OH) [33,62,63], and cause damage to different macromolecules mainly proteins, carbohydrates, lipids, and nucleic acids [48,64]. In addition, ROS increases the peroxidation of lipids, affecting the structure of cell membranes and disturbs their vital functions [65,66]. Additionally, Nrf2 is well known as the main regulator for the mRNA expression of the different antioxidant genes. It is increased when ROS is overproduced, leading to further DNA damage and increased cancer risk [67]. It is well known also that hydrogen peroxide (H2O2) accumulates in the liver and kidney when SOD decreases, resulting in the decrease in CAT which converts H_2O_2 to O_2 and H_2O and prevents additional oxidative damage [68,69]. Previous studies indicated that the surface chemistry of TiO_2-NPs plays a vital role in their toxicity in size and time-dependent, since it affects TNF-α release, inflammatory responses, and neutrophil-attracting chemokines [70,71].

TiO_2-NPs exposure also resulted in severe disturbances in mRNA expression of the antioxidant enzymes GPx, CAT, and SOD. The mRNA expressions of these enzymes were down-regulated in rats that received TiO_2-NPs. Taken together, the down-regulation of these antioxidant enzymes and the elevation in cytokines suggested the overproduction of ROS after the ROS overproduction after exposure to TiO_2-NPs. This ROS activates some receptors resulting in the activation of different signaling pathways accountable for reducing the antioxidant enzymes by ROS formation [29]. Also, animals treated with TiO_2-NPs showed an increase in the percentage of DNA fragmentation in the hepatocytes which confirms the overproduction of hydroxyl radical, as the main destructive species that increase DNA damage [72].

The examination of liver sections revealed serious changes in the hepatic structure and confirmed the disturbances in biochemical and cytogenetic results and coincidence with the literature [73,74]. These authors found disorganization of hepatic cords, hepatocellular necrosis, and micro-and macro-vascular steatosis. These alterations are It is mainly associated with oxidative stress around the central vein [75]. It was suggested that there is a strong relationship between oxidative damage and tissue hypoxia in different tissues and organs [76,77]. Additionally, it is well known that Kupffer cells in hepatic tissue are the cells most damaged during oxidative stress due to localization near the portal region [78].

Our results also showed that oxidative damage is the main manifestation of TiO_2-NPs toxicity, and thus, the supplementation with antioxidants may be a useful tool to protect against TiO_2-NPs-induced oxidative damage. EOs such as TO and BO are rich in several bioactive compounds with powerful antioxidant activity [79,80]. Due to several limitations in their application, the emulsifying of EOs may be useful to face such limitations since it protects the bioactive constitutes against different technological processes, enhances their stability and bioavailability, and controlled their release [64,81].

In this study, the protective role of TO and BO and their nanoemulsions (TON and BON) were evaluated against the oxidative damage of TiO_2-NPs. Previous studies showed that TO is rich in bioactive compounds belonging to terpenes, terpenes derivatives, and phenols, and thymol and carvacrol are the major constituents [49,82-84]. Whereas, the BO was reported to be rich in terpene, terpene alcohol, sesquiterpenoids, and phenylpropanoids and the major constituents were linalool and methyl chavicol [45,85,86]. The current data also revealed that administration of TO plus BO or TON plus BON induced positive against TiO_2-NPs toxicity with no obvious toxicity or side effects. Furthermore, these agents exhibited remarkable improvement in the biochemical indices, serum cytokines, lipid profile, and the oxidative stress markers in animals administrated TiO_2-NPs suggesting the sturdy antioxidant properties of their bioactive compounds such as thymol and carvacrol in TO and linalool and methyl chavicol in BO [10,87,88]. In addition to their pro-oxidant properties which protect against the damage of DNA and suppress the generation of ROS [^9].

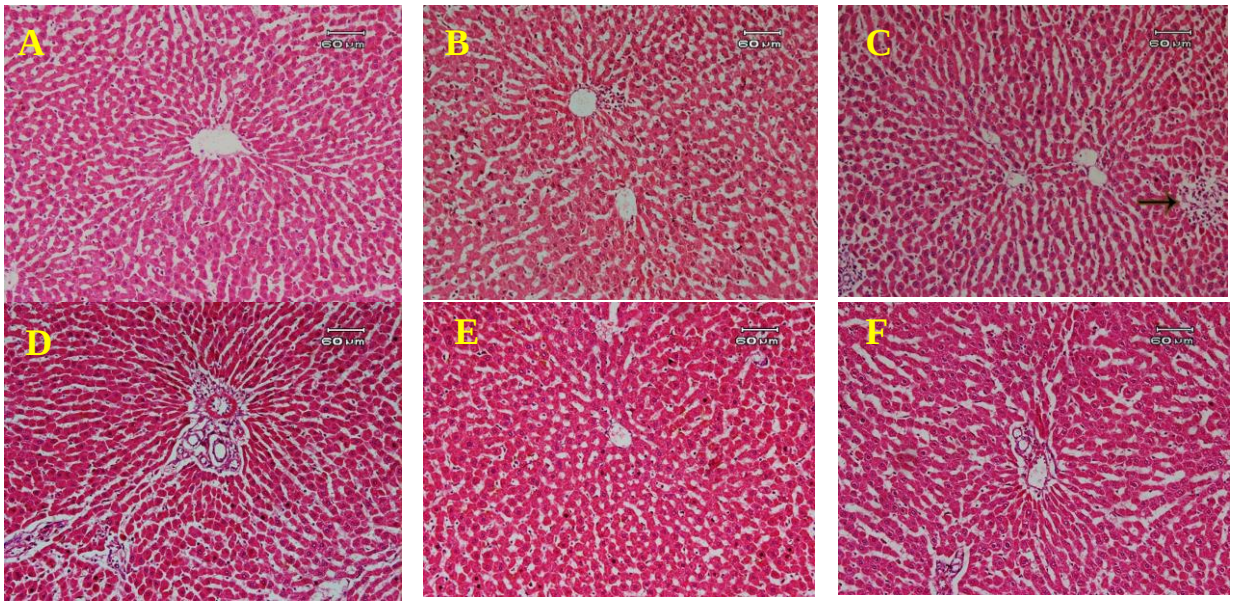


Fig. 3. Photomicrograph of the liver sections of (A) control rats showing normal structure of hepatic lobule, central vain, and blood sinusoid; (B) rats received TO plus BO showing normal liver architecture, slight dilatation of blood sinusoid, and few inflammatory cells; (C) rats received TON plus BON showing relatively normal histological pattern, some aggregation of inflammatory cells, some nuclei are pyknotic and peripheral chromatin clumping; (D) rats received TiO_2-NPs showing dilatation in portal tract, proliferation in the bile duct epithelial cells, the hepatocytes revealed vacuolar degeneration, the nuclei appear small in size and some of them showed pyknotic, necrotic, karyolysis and peripheral chromatin clumping; (E) rats received TiO_2-NPs plus TO and BO showing some improvements, the hepatic tissue relatively normal around central area, slight vacoular degeneration, while the nuclear damage in the form of binucleation, pyknosis, karyolysis were still presented; (F) rats received TiO_2-NPs plus TON and BON showing more improvements represented marked diminution of fibrosis, the structure of hepatic tissue almost normal around the portal area, and slight diffusion of inflammatory cells. (H x & E x 200)

Methyl chavicol in BO can block the voltage-activated sodium channels and stimulate the anti-inflammatory effects via the suppression of leukocyte migration and the stimulation of macrophages phagocytosis [٩٠,91]. Additionally, γ-γ-Muurolene is also a major compound in the BO and has antioxidant and anti-inflammatory properties [92,93], and β-elemeneas can regulate the oxidative stress along with several inflammatory factors cytokines including TNF-α, IFN, TGF-β, and IL-6/10 [94]. Moreover, β-elemeneas also induces apoptosis of cancer cells and inhibits the P21-activated kinase1 (PAK1) signaling pathway [95,96], so it is applied for the therapeutic of cancer in several organs such as the liver [97], stomach [96], lung [98], ovary [٩٩], breast [100], and brain [١٠١].

In addition, these oils contain phenolic compounds known for their potent antioxidant activities and decrease the plasma TriG, Cho, and LDL-Cho along with their radical scavenger activity [102]. These oils enhanced the activity of the endogenous enzymes (CAT and SOD) which are the main players in hepato-protection [103]. Despite the well-known antioxidant activity of TO and BO, the current data showed a synergistic activity of the combination of both EOs either in the crude or nanoemulsion. According to Olszowy-Tomczyk [104], this may be due to (1) the formation of the stable intermolecular complexes between the antioxidants compounds, which induce higher activity than the parent compounds, (2) the formation of both adducts and new phenolic compounds with greater antioxidant activity than the mixture of parents constitutes, (3) the differences in the solubility of different antioxidants and (4) the unexpected interaction between different compounds. Moreover, the occurrence of minor components in the EOs play a role in their antioxidant properties and acting in a synergetic mechanism, hence, the outcome antioxidant properties may be resulted from the interactions between many components present in the EOs [105]. In general, we can postulate that the mode of action of these EOs could be via the radical scavenging activity, the inhibition of certain enzymes accountable for the generation of ROS, and the chelation of iron which promote the radical reactions [٤٨,106]. Other mechanisms may be involved, such as the interference of antioxidants with xenobiotic-metabolizing enzymes that inhibit the mutagens/carcinogens, regulating DNA repair and modulating the gene expressions [107]. Recent data proved that the combination of lower

concentrations; the EOs is an effective and promising tool to improve their biological activity and reduce the impacts of high doses [108].

Moreover, there is a growing interest to use a combination of EOs that shows synthetic effects [1،9]. The synergistic effect enhances the antioxidant activity and reduces the doses required for the combined pharmaceuticals [11،,111]. The mechanism of the synergistic effects is mainly due to the nature of multi-component of the combination. In the current study, the TO and the BO have different compounds which probably induce synergistic effects on multiple biochemical processes in the cell and enhance the effectiveness of each other [112]. Additionally, the nature of the combination may improve the bioavailability of different combined agents [113]. It is well documented that EOs are rich in sulfur-containing compounds, terpenoids, and phenolic compounds, mainly thymol, carvacrol, linalool, and eugenol, which are responsible for EOs scavenging activity [114].

Moreover, the nonvolatile constituents such as caffeic acid, rosmarinic acid, quercetin and carnosol, and quercetin have a potent antioxidant activity due to their special radical chemistry [115]. Additionally, phenolic components can scavenge the reactive species, provide hydrogen atoms and/or donate electrons from the superoxide, peroxyl, and hydroxyl groups, and act as chain-breaking antioxidants [116]. The combinations of phenolic monoterpenes (carvacrol and thymol) and phenylpropanoid (chavicol and eugenol) with other components were found to increase the bioactivities, including antioxidant, antiherbivore, antimicrobial, and other pharmaceutical activities [117].

The present outcomes revealed that TON plus BON was more efficient than the TO plus the BO due to the antioxidant activity of WPI used in the preparation of emulsion. The WPI is rich in several amino acids which act as potent antioxidants, such as bovine serum albumin, cysteine, α- and β-lactoglobulin [118], and showed potent hepatoprotective against liver damage [25,119]. In addition to cysteine, the responsible for the regeneration of GSH, the endogenous antioxidant responsible for peroxide detoxification [120].

Conclusion

The current data indicated that exposure to TiO_2-NPs resulted in damage to different organs via the enhancement of oxidative stress. These particles disturb the biochemical indices, the lipid profile, the serum cytokines, the antioxidant/oxidant markers, mRNA expression of antioxidant genes, DNA fragmentation, and the histological structure of the liver. Both TO and BO in the crude form or in the nanoemulsion form could prevent these disturbances and prevent the oxidative damage of TiO_2-NPs; however, the nanoemulsion form was more effective. These outcomes indicated the safety eco-friendly of the TON and BON. The protective role of these nanoemulsions is mainly due to the synergistic effect and indicated that the nanoemulsion form enhanced the antioxidant efficiency of the EOs, expanding their dispersibility and fostering their controlled delivery. Moreover, the antioxidant property of the WPI used in the preparation of nanoemulsion is considered an additional mechanism for enhancing the antioxidant activity of TON and BON. Therefore, TON and BON are eco-friendly agents suitable for natural antioxidant additives to be used in the food and pharmaceutical sectors.

Acknowledgments

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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