Research Article

Genetic, Biochemical and Histological Studies to Investigate the Effect of *Punica granatum* and *Citrus aurantium* Peel Extracts on Male Rabbit Fertility

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Accepted on: 02-06-2016; **Finalized on:** 31-07-2016.

ABSTRACT

The idea of this study was carried out to investigate the link between nutritional supplements rich in antioxidants and fertility. *Punica granatum* and *Citrus aurantium* peel extracts effects on fertility were studied in New Zealand rabbit ducks. Study was conducted by investigation of semen physical characters, hormonal analysis, gene expression of Nerve growth factor (NGF) gene and their receptors NTRK1 and NGFR and histological examination. The experiment was carried out using 20 sexually mature New Zealand white male rabbit. The rabbits were divided into 4 groups; the first group received drinking distilled water and served as negative control. The second group was received a dosage of 5mg/kg of sildenafil citrate and served as reference drug. The third and fourth groups were received aqueous extracts of *Punica granatum and Citrus aurantium* peel at the dosage of 200 mg/kg daily for 28 days. Hormonal analysis revealed both extracts significantly increase the levels of testosterone, follicular stimulating hormone and LH. *Punica granatum* significantly decreased the sperm physical characters, whereas, Citrus *aurantium* showed non-significant differences. *Punica granatum* and *Citrus aurantium* peel extract increased the expression of nerve growth factor (NGF) gene and their receptors (NTRK1& NGFR) gene. In addition, histological examination showed that *Citrus aurantium* increases the efficiency of testicular tissue. However, the both extracts revealed moderate alteration in liver tissue. Hence, we can concluded that *Citrus aurantium* have the aptitude to improve the fertility of rabbit bucks more than *Punica granatum.* Nevertheless, it could not be suggested for a long period due to their negative relation to hepatocytes histological alteration.

Keywords: Punica granatum, Citrus aurantium, peel extract, fertility, hormonal analysis, gene expression, histology, rabbit.

INTRODUCTION

The shortage of good quality feeds needed to maintain livestock growth during the waterless season has been a main challenge to the industry in the developing countries. Thus, agro-industrial by-products are being evaluated to admission their nutritive potential to sustain livestock productivity. Agro-industrial by-product peel meals have been finding to be a source of protein similar with maize.[1] Fruit and vegetable processing by-products are hopeful sources of important substances such as phytochemicals (carotenoids, phenolics, and flavonoids), antioxidants, antimicrobials, vitamins, or nutritional fats that own favorable technological activities.[2] Its have been used in animal nourishment as the chief feed ingredients and their effect on animal performance has been studied.[3,4] Recent facts shows that fruit and vegetable processing by-products can be efficiently used in livestock feed as functional ingredients for the production of improved quality food products.[5]

The bitter orange (*Citrus aurantium*) is usually used as a medical and nutritional supplement.[6] The orange is an important source of vitamin C and polyphenolic compounds. The main phenolic compounds present in the orange include hydroxycinnamic acids (HCA) and flavonoids.[7] The antioxidant concentrations differ among the different parts of the orange. Citrus peels contain a higher concentration of antioxidant substances compared to the edible portions.[8] Sour orange peel composed of flavonoids, Vitamin C, Carotene and Pectin. Flavonoids are aromatic secondary plant metabolites, which have been recognized as vital due to their physiological and pharmacological function and their health benefits.[9] Citrus flavonoids have a broad range of therapeutic properties, including anti-inflammatory, antihypertensive, diuretic, pain-relieving and hypolipidemic properties.[10] In addition, it is attracting more attention as anti-carcinogenic agent due to their pharmacological activity as free radicals scavengers.[11]

Pomegranate *(Punica granatum)* is an old fruit cultivated in Egypt and belongs to the Punicaceae family.[12] The pomegranate description as healthy fruit belongs to its integrity due to the polyphenols high content, vitamins C and B6, minerals, and fiber. Pomegranates have been useful in treating a wide variety of diseases including; atherosclerosis, coronary heart disease, prostate cancer and male infertility.[13]

Pomegranate peel is a rich antioxidant contains a high content of quercetin and kaempferol, flavond, diglycoside, ellagic acid tannin and organic acids.[14,15] Pomegranate components have attracted great attention for their wound-healing properties, immune-modulator

activity and antioxidative capacities[14,16,17], treatment and prevention for cancer[18], cardiovascular disease[19], diabetes[20], antimicrobial[21] and erectile dysfunction.[22] Recent studies revealed that antioxidant contents of pomegranate peel improve lambs health and performance.[23,24]

Nerve growth factor (NGF) is a soluble protein that secreted by various tissues in the body. The nerve growth factor (NGF) biological effects are mediated by two classes of receptors, a high-affinity specific 140-kDa trans-membrane neurotrophic tyrosin kinase receptor (NTRK1) and a low-affinity 75-kDa receptor (NGFR, nerve growth factor receptor) belonging to the family of tumor necrosis factor receptor.[25] The wide distribution and differential expression pattern of NGF system within specific cellular types of rabbit male sex organs raises interesting about the potential functions of this neurotrophin in the regulation of reproductive function. On the basis that NGF and NTRK1 specially localized in the germinal and endocrine cells of the male gonads, it argued that NGF exerts an autocrine and/or paracrine function through testicular maturity and spermatogenesis.[26] Several studies have shown that NGF influences the reproductive function of both males and females.[27,28]

Reactive oxygen species (ROS) are molecules that are highly disruptive to cellular function and have free radicals. ROS produced in the testis as a usual physiological event motivate the oxidation and DNA damage.[29] The sperm plasma membrane has polyunsaturated fatty acids, which are sensitive to peroxidative damage. The lipid peroxidation demolishes the construction of the lipid in the membranes of spermatozoa, hence, reduces the sperm's motility and results in defects to membrane integrity.[30,31] Antioxidants are compounds that help to control ROS and lipid peroxidation.[32] Meanwhile, Arbo[33] reported the anti-estrogenic potential of *C. aurantium*. Hence, it can be linked with fertility in female animals. However, no study has so far reported either positive or negative effects of *C. aurantium* on male fertility. Based on these conclusions the main purpose of the present study is to estimate the effect of *Punica Granatum and Citrus aurantium* Peel Extracts on, sex hormones, semen physical characters, the enhancement of the expression of NGF gene and its receptors (NTRK1 and NGFR) and the histological changes in testis of male rabbit.

MATERIALS AND METHODS

Aqueous Extract Preparation

The peels of citrus and pomegranate were thoroughly washed with tap water, dried in room temperature, powdered in an electrical blender.

The aqueous extracts were obtained by the percolation method. One kilogram of each powder was extracted with 3liter of distilled water and left to stand for 72 hours at room temperature. The extract was filtered with Whatman No. 1 filter paper. The crude aqueous extract was concentrated using rotary evaporator under reduced pressure at 45°C then the concentrated extracts were lyophilized and kept at -20°C. The extract was mixed with distilled water to obtain concentrations.

Acute Oral Toxicity

The acute toxicity test for plant extracts was estimated to evaluate any possible toxicity. Animals were tested by administering different extracts doses by increasing or decreasing the dose, according to the response of animal.[34] The dosing patron was 500, 1000, 1500, 2000, 2500, 3000, 3500 and 4000 mg/kg body weight for aqueous extracts of *punica granatum* and *citrus aurantium* peel, while control group received only the normal saline. All groups were observed for any gross effect or mortality during 48hr. Death of half of examined animals was observed at 2000 mg/kg b wt for both extracts.

Animals and Dosing

The experiments were carried out using 20 sexually mature New Zealand white male rabbit weighing 2.40 ± 0.08 kg. The animals were acquired from the research rabbit unit of agriculture experimental station, Faculty of agriculture, Cairo University. Rabbit bucks were housed in polyethylene boxes in a climate-controlled environment (25° ± 4°C, 55 ± 5% humidity) with a 12-h light/dark cycle. They were fed on commercial food pellets and drinking water *ad libitium*. The rabbits were divided into 4 groups of 5 rabbits each. The first group received drinking distilled water *ad libitium* and served as negative control. The second group were received a dosage of 5mg/kg of sildenafil citrate (Viagra tablets, German Remedies) and used as reference group. The third group was received aqueous extract of *punica granatum* peel at the dosage of 200 mg/kg. The fourth group was received aqueous extract of *citrus aurantium* peels at the dosage of 200 mg/kg. A calculated dose of the extract according to the initial body weight of the rabbits was administered orally to each rabbit at 8 a.m. daily for 28 days respectively. Body weights of the rabbits were measured weakly. This study was carried out following approval from the Ethical Committee on the Care and Use of Experimental Animals for Research of National Research Centre.

Serum Hormonal Investigation

The rabbits were starved for 12 h and blood samples were collected for biochemical evaluations. Blood samples were collected from the marginal ear vein of the rabbits into clean dry centrifuge tube and left to clot at room temperature.

Then samples centrifuged for 10 minutes at 3000 r pm to separate serum. Serum was carefully separated into dry clean Wassermann tubes, using a Pasteur pipette and kept frozen at (-20°C) until estimation of some biochemical parameters.

Testosterone levels were measured in serum by ELISA testosterone standard kits (Biocheck, Inc. Foster City CA,

USA) according to Tietz[35]. Estimation of serum thyroid hormones (T3, T4&TSH) was done by chemiluminescence immunoassay method according to Dermer[36]. Determination of LH and FSH hormones was done by ELISA method.

Biochemical Investigation

Total cholesterol and HDL-cholesterol were determined by the method of Stein[37]. Cholesterol-LDL was calculated according to Friedewald[38]. Triglyceride was measured in serum by the method of Wahelfed[39]. Total lipid was measured in serum by the method of Zollner and Kirsch[40]. Determination of aspartate and alanine aminotransferases (AST and ALT) enzyme activities: AST and ALT were measured in serum by the method of Reitman and Frankel[41]. Total protein was determined in serum according to the method of Bradford[42]. All kits were purchased from Biodiagnostic, Egypt.

Semen Physical Properties Investigation

After 8 wk of adaptation to experimental treatments, semen collection and evaluation was done on bucks of all experimental groups. Semen collected twice a week for five consecutive weeks by the same handler in all groups according to IRRG Guidelines standard procedure. **Sperm motility** was estimated by placing a drop of the suspension on a clean glass slide under the cover slip. Motile and immotile spermatozoa were counted under a microscope with phase contrast optics (100×) according to Seleem[43]. **Sperm viability** was assessed by nigrosin/eosin (N/E) staining procedure according to Bakst and Cecil[44]. About 500 spermatozoa in each smear were counted by microscopy (magnification 100 xs) and the proportion of viable spermatozoa was calculated on the total number of cells. **Sperm membrane integrity** was determined in semen samples in a 1:10 dilution of hypoosmotic swelling test (HOST) solution according to Moce[45] and incubated at 37°C for 30 min. After incubation, a 5µl semen sample drop was examined under a phase-contrast microscope 400-x magnification. The grades and percentages of spermatozoa with swollen heads and coiled tails were estimated. The swollen spermatozoa characterized by coiling of the tail were considered to have an intact plasma membrane. The nigrosin-eosin stained semen smears as used in live sperm counts were also utilized in determining the percentage of morphological abnormal spermatozoa, while the smear was examined with bright field microscopy at 400 -x magnification.

Expression of NGF Gene and its Receptors NTRK1 and NGFR

Isolation of total RNA

Total RNA was isolated from blood samples collected from the ear veins of male rabbits by the standard TRIzol® Reagent extraction method (cat#15596-026, Invitrogen, Germany).

Total RNA was treated with 1 U of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water. Purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1). Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT).

Reverse Transcription (RT) Reaction

The complete Poly(A) ± RNA isolated from male rabbits blood samples was reverse transcribed into cDNA in a total volume of 20 µl using RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An amount of total RNA (5µg) was used with a reaction mixture, termed as master mix (MM). The MM was consisted of 50 mM MgCl2, 5x reverse transcription (RT) buffer (50 mMKCl; 10 mMTris-HCl; pH 8.3; 10 mM of each dNTP, 50 µM oligo-dT primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M-MuLV reverse transcriptase.

The RT reaction was carried out at 25 °C for 10 min, followed by 1 h at 42 °C, and the reaction was stopped by heating for 5 min at 99 °C. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through quantitative real time-polymerase chain reaction (qRT-PCR).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd., Germany), 0.5 µL 0.2 µM sense primers, 0.5 µL 0.2 µM antisense primer (Table 1), 6.5 µL distilled water, and 5 µL of cDNA template.

The reaction program was allocated to 3 steps. First step was at 95.0°C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 steps: (a) at 95.0°C for 15 sec; (b) at 55.0°C for 30 sec; and (c) at 72.0°C for 30 sec. The third step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 sec up to 95.0°C. At the end of each qRT-PCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers. Each experiment included a distilled water control.

The quantitative values of RT-PCR (qRT-PCR) of the expression of nerve growth factor (NGF) gene, cognate receptors neurotrophic tyrosine kinase receptor type 1 (NTRK1) gene and nerve growth factor receptor (NGFR) gene were normalized against the 18S co amplified product expression. At the end of each qRT-PCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers.

The amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formulae

of Bio-Rad Laboratories Inc. Bio-Rad Laboratories Inc. Real-Time PCR Applications Guide: Ef = $10^{-1/slope}$ Efficiency (%) = Ef = $10^{-1/slope}$ Efficiency (%) = (Ef – 1) x100.

The relative quantification of the target to the reference was determined by using the ΔCT method if E for the target (Cyp19, LH and FSH) and the reference primers (β-Actin) are the same.

Ratio $_{(reference/\ target\ gene)}$ = Ef $^{CT\ (reference)\ –\ CT(target)}$

Histopathological Assay

Samples of liver and testes of each rabbit were dissected out and quickly fixed in 10% buffered formol saline then processed for preparation of 5µm-thick paraffin sections. Sections were stained with Haematoxlin and eosin stain for histopathological examination.

Statistical Analysis

Results were expressed as the mean ± standard error of the mean (SEM).

Data for multiple variable comparisons were analyzed by one-way analysis of variance (ANOVA).

For the comparison of significance between groups, Duncan's test was used as a post hoc test according to the statistical package program (SPSS version 17.0). All p values are two-tailed and p≤ 0.05 was considered as significant for all statistical analysis in this study.

RESULTS

Body Weights

Data in Table 2 revealed that there were no significant differences in rabbit body weight of *Punica Granatum and Citrus Aurantium* groups and the control through the period of treatment.

While, Sildenafil citrate showed significant increase in the body weights at 3 and 4 week than those of peel extracts and control groups.

Table 1: Primers for NGF, NTRK1, NGFR and 18S used as internal standard for RT-PCR quantification

Gene	Primers (5'-3')	Product size (bp)
NGF	F-CAACAGGACTTACAGGAGCA R-GCCTTCCTGCTGAGCACGCA	369
NTRK1	F-GGACAACCCTTTCGAGTTCA R-GAAAAGGCAGGCAAAGACAG	157
NGFR (p75)	F-GCCTACATCGCCTTCAAGAG R-TCTGGCTGTCCACAGAGATG	136
18S	F-TCAAGAACGAAAGTCGGAGGTT R-GGACATCTAAGGGCATCA	489

Table 2: Effect of *Punica granatum* and *Citrus aurantium* and Sildenafil citrate on body weight of rabbit bucks.

Groups	Rabbit bucks body weights (g)			
	Week 1	Week 2	Week 3	Week 4
Control	2326.70^{a}±121.399	2323.3^{a}±124.70	2300.00^{b}±113.37	**2300.00^{b}±131.65**
Sildenafil citrate	2328.57^{a}±105.004	2557.00^{a}±120.13	2759.86^{a}±101.06	**2879.00^{a}±100.65**
Punica granatum peel	2360.00^{a}±113.81	2286.66^{a}±112.63	2360.000^{b}±94.38	**2365.00^{b}±138.41**
Citrus aurantium peel	2500.00^{a}±81.16	2500.00^{a}±77.46	2400.00^{b}±73.03	**2495.00^{b}±75.00**

Data are expressed as the mean ± standard Error (SE). Different superscripts within the same column designate significant differences (p≤0.05).

Table 3: Effect of Punica granatum and Citrus aurantium peel aqueous extracts on testosterone FSH and LH hormones levels in rabbit bucks.

Group	Testosterone (ng/dl)	FSH (mIµ/ml)	LH (mIµ/ml)
Control	798.500^{d} ± 0.288	1.027^{c} ± 0.009	0.130^{c} ± 0.0002
Sildenafil citrate	1193.000^{c} ± 0.577	0.513^{d} ± 0.009	0.130^{c} ± 0.0003
Punica granatum peel 200mg/kg	1238.000^{b} ± 0.577	1.122^{b} ± 0.009	0.149^{b} ± 0.0003
Citrus aurantium peel Ext., 200mg/kg	1305.000^{a} ± 0.577	1.495^{a} ± 0.009	0.153^{a} ± 0.0005

Data are expressed as the mean ± standard error (SE). Different superscripts within the same column designate significant differences (p≤0.05).

Table 4: Effect of *Punica granatum* and *Citrus aurantium* peel aqueous extracts on thyroid hormones in rabbit bucks

Group	T3 (pg/dl)	T4 (ng/dl)	TSH (µlU/ml)
Control	$4.480^{b} \pm 0.075$	$1.310^{b} \pm 0.006$	$0.037^{a} \pm 0.008$
Sildenafil citrate	$5.400^{a} \pm 0.057$	$1.390^{a} \pm 0.006$	$0.020^{a} \pm 0.005$
Punica granatum peel	$4.250^{c} \pm .028$	$1.240^{d} \pm 0.005$	$0.013^{a} \pm 0.005$
Citrus aurantium peel	$3.930^{d} \pm .011$	$1.290^{c} \pm 0.005$	$0.023^{a} \pm 0.008$

Data are expressed as the mean ± standard Error (SE). Different superscripts within the same column designate significant differences (p≤0.05).

Table 5: Impact of *Punica granatum* and *Citrus aurantium* peel aqueous extracts on lipid profile functions in rabbits bucks

Group	Total cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	Triglyceride (mg/dl)	Total lipid (mg/dl)
Control	$124.233^{b} \pm 0.36$	$78.50^{c} \pm 0.26$	$35.433^{b} \pm 0.21$	$362.600^{b} \pm 0.11$	$2446.233^{b} \pm 34.15$
Sildenafil citrate	$139.533^{a} \pm 0.54$	$73.333^{d} \pm 0.33$	$66.200^{a} \pm 0.23$	$578.900^{a} \pm 0.51$	$2666.433^{a} \pm 56.14$
Punica granatum peel	$125.6^{b} \pm 0.23$	$80.50^{b} \pm 0.10$	$34.980^{b} \pm 0.36$	$316.600^{c} \pm 0.23$	$2090.663^{c} \pm 27.42$
Citrus aurantium peel	$117.100^{c} \pm 0.55$	$90.983^{a} \pm 0.06$	$26.023^{c} \pm 0.33$	$261.667^{d} \pm 0.33$	$1428.100^{d} \pm 12.88$

Data are expressed as the mean ± standard Error (SE). Different superscripts within the same column designate significant differences (p≤0.05). HDL: High-density lipoprotein; LDL: low-density lipoprotein.

Table 6: Impact of *Punica granatum* and *Citrus aurantium* peel aqueous extracts on liver functions in male rabbits

	Total protein	Glutamic oxalacetic Transaminase (IU/L)	Glutamic pyruvic Transaminase IU/L
Control	$5.03^{b} \pm 0.08$	$134.333^{d} \pm 0.37$	$175.533^{b} \pm 0.03$
Sildenafil citrate	$4.70^{c} \pm 0.05$	$166.433^{a} \pm 0.29$	$185.1^{a} \pm 0.40$
Punica granatum peel	$4.93^{bc} \pm 0.02$	$142.667^{b} \pm 0.33$	$152.967^{c} \pm 0.52$
Citrus aurantium peel	$5.70^{a} \pm 0.11$	$137.000^{c} \pm 0.57$	$145.000^{d} \pm 0.34$

Data are expressed as the mean ± standard Error (SE). Different superscripts within the same column designate significant differences (p≤0.05).

Table 7: Effect of Sildenafil citrate, *Punica granatum* and *Citrus aurantium* peel aqueous extracts on semen physical characters in male rabbit.

	Control	Sildenafil citrate	Punica granatum	Citrus aurantium
Sperm motility	$89.20^{a} \pm 1.64$	$75.70^{b} \pm 1.21$	60.60 ± 1.67^{c}	86.30 ± 0.87^{a}
Sperm Membrane integrity	$93.50^{a} \pm 1.06$	83.20 ± 1.45^{b}	61.60 ± 0.97^{c}	91.30 ± 0.93^{a}
Live sperm	$90.50^{a} \pm 2.20$	$85.50^{ab} \pm 1.19$	67.90 ± 1.49^{c}	84.90 ± 1.83^{b}
Dead sperm cells	$9.50^{c} \pm 2.20$	$14.50^{b} \pm 1.19$	32.10 ± 1.49^{a}	15.10 ± 1.84^{b}
Normal morphology (%)	$81.40^{a} \pm 1.71$	$80.30^{a} \pm 2.78$	63.10 ± 1.54^{b}	77.20 ± 1.59^{a}
Abnormal morphology (%)	$18.60^{b} \pm 1.71$	19.70 ± 2.78^{b}	36.90 ± 1.53^{a}	22.80 ± 1.60^{b}

Data are expressed as the mean ± standard error (SE). Different superscripts within the same column designate significant differences (p≤0.05).

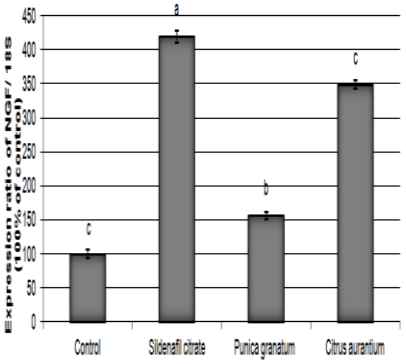

Figure 1: The relative expression of NGF gene in blood samples of male rabbits supplemented with *Citrus aurantium* and *Punica granatum* peel extracts. Rate values in the same column with different superscript differ significantly (*P*≤ 0.05).

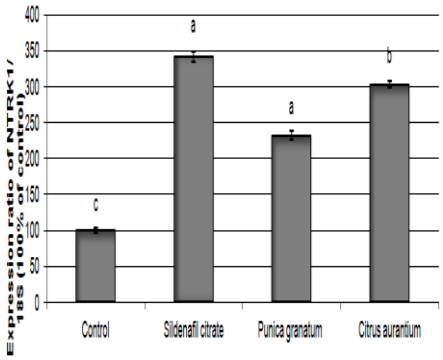

Figure 2: The relative expression of NTRK1 gene in blood samples of male rabbits supplemented with *Citrus aurantium* and *Punica granatum* peel extracts. Rate values in the same column with different superscript differ significantly (*P*≤0.05).

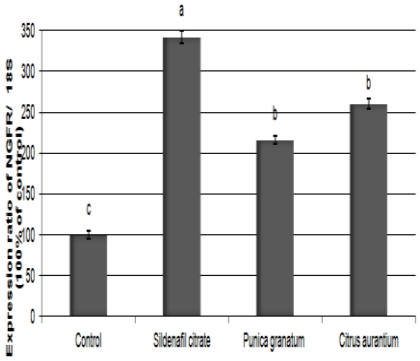

Figure 3: The relative expression of NGFR gene in blood samples of male rabbits supplemented with *Citrus aurantium* and *Punica granatum* peel extracts. Rate values in the same column with different superscript differ significantly (*P*≤ 0.05).

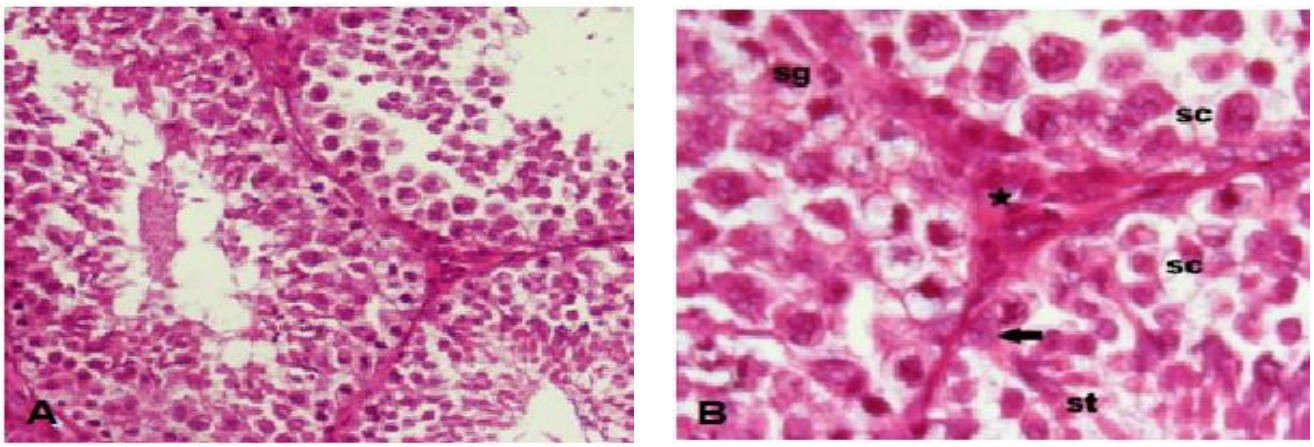

Figure 4: Section of control testis of rabbit showing: (A) normal structure of seminiferous tubules, (B) High power showing the different stages of spermatogonic cells in the seminiferous tubules, spermatogonia(sg), spermatocyte cell (sc), spermatid (ST) as well as sertoli cells (arrow) and the interstitial cells (star) (Hx&E x400).

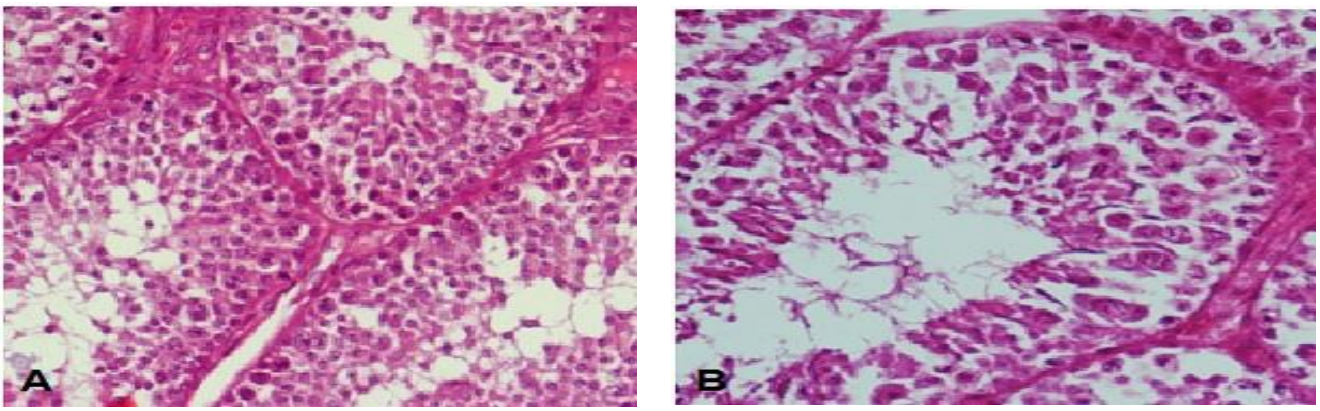

Figure 5: Section of rabbit testis treated with: **A**) *Punica granatum* peel extract showing normal structure of seminiferous tubules containing all developmental stages of spermatogenesis. The shape of the tubules oval, and without alterations, Cytoplasmic swelling of spermatogonia, or showing no abnormality. B) *Citrus aurantium* peel extract showing the different stages of spermatogonic cells in the seminiferous tubules , spermatocyte cells and spermatid appear more regularly and numerous (Hx&E x400).

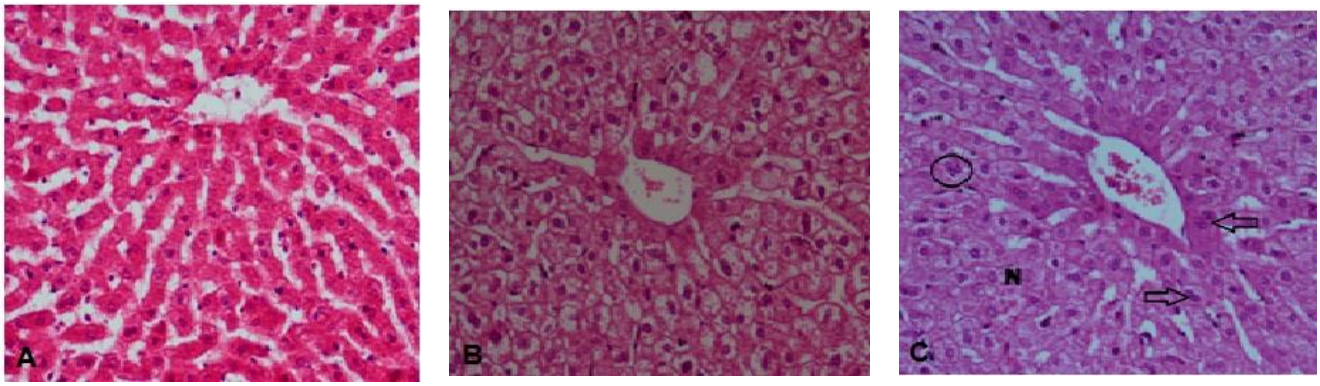

Figure 6: A) Section of control liver of Rabbit showing cords of hepatocytes radiating from the central vein, and blood sinusoids. B) Photomicrograph of liver section of Rabbit treated with *Punica granatum* peel extract revealing vacuolar and ballooning of some hepatocytes degeneration of nuclei (necrosis, pyknosis and karyolysis) and binucleation. C): section of rabbit liver treated with *Citrus aurantium* peel showing dilation in central vain and blood sinusoids , signs of degeneration in some hepatocytes in the form of ballooning of cytoplasm and necrosis(N), pyknosis ,keryolsis in addition to increase in binucleation (arrowe),and trinuclei also present. (ring) .(Hx&E x400).

Biochemical Results

Hormonal analysis in Table 3 revealed significant increase *(p≤0.01)* in serum testosterone, FSH and LH levels of *Punica granatum* and *Citrus aurantium* (200mg/kg) treated rabbit bucks than control groups. In addition, data revealed that Citrus *aurantium* peel aqueous extract increased the hormonal levels more than the *Punica granatum* extract. Meanwhile, *Punica granatum* and *Citrus aurantium* significantly decreased the levels of T_3 and T_4 serum hormones *(p≤0.01)* than those of control groups as show in Table 4. While, TSH show none significant decreased in both *Citrus aurantium* and *punica granatum* treated animals than control.

As regards to lipid profile functions, data in Table 5 showed no significant variation in the levels of total cholesterol and LDL in control and *Punica granatum* peel extract treated animals. Whereas, there was significant increase in HDL level than control. Meanwhile, *Punica granatum* peel extract significantly decreased the levels of triglyceride and total lipid (p≤0.01) as compared to control groups. *Citrus aurantium* significantly decreased the levels of cholesterol, LDL, triglyceride and total lipid (p≤0.01) as compared to control; while increased the HDL serum level.

Total protein, GOT and GPT in the control, *Punica granatum* and *Citrus aurantium* peel aqueous extracts treated rabbit bucks are show in Table 6. Data exhibited that *Punica granatum* peel extract significantly decreased the activity of GOT and GPT (p≤0.01) and none significantly decreased the total protein level as compared to negative control. Whereas, the *citrus aurantium* peel extract significantly increased the total protein level and decreased GOT and GPT serum levels (p≤0.01).

Semen Physical Properties

Semen analysis results were presented in Table 7. Results revealed that the *Punica granatum* peel extract oral gavages at dose of 200 mg/kg for 28 days significantly decreased the sperm motility, membrane integrity, live and the percentage of normal morphological sperm (p≤0.01) as compared to control and sildenafil citrate groups.

Whereas, there was no significant differences in the semen physical characters of rabbit bucks administrated Citrus *aurantium* peel extract at dose of 200 mg/kg and control group.

While, Citrus *aurantium* peel extract significantly increased the sperm motility, membrane integrity and live number (p≤0.01) as compared to sildenafil citrate.

Effect of Punica granatum and Citrus aurantium on the expression NGF gene and its receptors NTRK1 and NGFR

The effect of *Punica granatum* and *Citrus aurantium* peel extract on the expression of nerve growth factor (NGF) gene, cognate receptors neurotrophic tyrosine kinase receptor type 1 (NTRK1) gene and nerve growth factor receptor (NGFR) gene in blood samples of adult male rabbits is summarized in Figures 1-3.

The expression levels of NGF gene were increased significantly (P≤0.05); by 348.8%) in *Citrus aurantium* treated new Zealand white male rabbit compared with control group, however, the increase of the expression level of this gene was insignificantly in *Punica granatum* treated male rabbit (Figure 1).

Moreover, treatment of male rabbits with *Citrus aurantium* and *Punica granatum* increased significantly (P≤0.05; by 303.2 and 232.2%, respectively) the expression of NTRK1 gene compared with control group (Figure 2). Furthermore, supplementation of male rabbits with *Citrus aurantium* and *Punica granatum* extracts increased significantly (P≤0.05; by 260 and 216.3) the levels of NGFR gene compared with control group (Figure 3). Meanwhile, salidofil citrate significantly increased the expression of NGF, NGFR and NTRK1 genes (P≤0.01) as compared to *Citrus aurantium* and *Punica granatum.*

Histopathological Results

The microscopic examination of control section of rabbit testis showed normal and healthy structure of seminiferous tubules, spermatogonia on the basement membrane, spermatocytes, density of spermatids, spermatozoa and sertoli cells can be observed in between the germ cells, as well as the leyding cells in interstitial area (figure 4). In *Punica granatum* peel extract treated group, testes revealed normal architecture of seminiferous tubules, cytoplasmic swelling of spermatogonia, high density of spermatid and well development spermatozoa, or showed no abnormality (Figure 5A). In *Citrus aurantium* peel extract treated group, the rabbits testes showed the different stages of spermatogonic cells in the seminiferous tubules, spermatocyte cells and spermatid appear more regularly and numerous (Figure 5B).

The microscopic examination of liver tissues of rabbit treated with *Punica granatum* peel extract revealed vacuolar and ballooning of some hepatocytes, degeneration of nuclei (necrosis, pyknosis and karyolysis) in addition to bi-nucleation (Figure 6B) comparing to control group (Figure 6A). *Citrus aurantium* peel extract treated rabbit liver revealed dilation in central vain and blood sinusoids, signs of degeneration in some hepatocytes in the form of ballooning of cytoplasm and necrosis, pyknosis, keryolsis in addition to increase in bi-nucleation, and tri-nuclei also present (figure 6C).

DISCUSSION

A familiar concept is that inadequate vitamins intake can cause venomous effects on spermatogenesis and construction of normal sperm. On the other hand, sufficient consumption of vitamins and natural antioxidants can protect sperm DNA from oxidative stress and hence it improves male fertility.[46] Fruit and vegetable processing by-products are promising sources of valuable substances such as phytochemicals, antioxidants, antimicrobials, vitamins, or dietary fats that possess nutritional properties.[2] In the present study, the effect of *Punica granatum* and *Citrus aurantium* Peel Extracts on rabbit male fertility was investigated. None of *Punica granatum and Citrus aurantium* peel extracts had significant effect on body weights of the male rabbits during four weeks when compared to the control group. This result was in consent with the result of Dkhil[47]. They indicated that pomegranate peels extract not affect the body weights of male rats.

Testosterone is the major male gonad hormone produced by the interstitial cells of the Leydig in the testis. Testosterone also helps in maintain body shape and increasing muscle mass and potency.

The raise in testosterone should improve mating performance and maintenance of spermatogenesis. Follicular stimulating hormone (FSH) stimulates spermatogenesis in the Sertoli cells and luteinizing hormone LH stimulates synthesis and release of testosterone in the Leydig cells.[48] Testosterone may also support male sexual activities by increasing dopamine release in the medial preoptic area and potentiating nitrergic neurotransmission.[49] Hormonal investigations revealed that *Citrus aurantium and Punica granatum* peel aqueous extracts increased the serum levels of testosterone, LH and FSH in male rabbits. This may be for the reason that *Citrus aurantium and Punica granatum* are rich of vitamin C, flavonoids, monoterpens and coumarins that are a kind of phytoestrogen. Goli[50] showed that used fruit extracts as antioxidant consequently thwart oxidative stress and improved secretion of testosterone and LH hormone in rats. Since, metabolism of many compounds by genital cells causes an increase in the level of the free radicals that can react with oxygen cause an increase in ROS production.[31] The results were in agreement with Komili[51] they showed that sour orange fruit essence increased the serum level of testosterone, LH and FSH in diabetic rats. In addition, Goli et al.[50] found that pomegranate peels and juice caused rise in the male sex hormones suggesting that pomegranate peels and juice may contain some biologically active ingredients that may be active against oxidative stress. According to study of Mahmoodi[9], flavonoids have vital pharmacologic property in preventing oxidation of lipoproteins with low molecular weight (LDL). This appeared in the present study where *Citrus aurantium* significantly decreased the cholesterol, LDL, triglyceride and total lipid levels.

Spermatozoa are vulnerable to peroxidative damage due to the high concentration of polyunsaturated fatty acids that are involved in regulation of sperm maturation, spermatogenesis, capacitation, acrosome reaction and eventually in membrane fusion, and low antioxidant capacity. This in turn results in axonemal damage, decreased sperm viability and increased midpiece morphological defects, and can completely inhibits spermatogenesis.[30,32] In the present study, semen analysis revealed that *Citrus aurantium* showed no significant differences in semen physical properties than control, while, *Punica granatum* showed negative effects. Khakpour[52] observed that *Citrus aurantium* extract was significantly increased the semen concentration and diminished the sperm motility in mice. It seems that *Citrus aurantium* has directly affected the spermatogenic cells. The study has also shown that the number of spermatogonia cells and primary spermatocytes were increased. S´anchez-Lamar[53] revealed that *Punica granatum* extracts significantly increased the percentage of sperm abnormalities and suggests that the random use of pomegranate fruit extract could represent a genetic risk for genome stability in the descendant generation of exposed individuals.

Nerve growth factor (NGF) is essential in promoting the development of the testis and the differentiation, maturation, and movement of the spermatozoon.[54] In the present study, *Citrus aurantium* increased significantly the NGF expression, while, the effect of *Punica granatum*

was insignificantly. This finding was confirmed with the semen analysis, since the increase in NGF maintain the semen physical characters of male rabbit in normal limit as control. While, the expression of NGF in *Punica granatum* group was less than in *Citrus aurantium,* therefore it decreased the sperm motility, membrane integrity, live and the percentage of normal morphological sperm. Evidence exists that NGF modulates certain sperm functions such as sperm motility and sperm viability.[55,56]

Concerning histological results, testes treated with *Punica granatum* revealed normal construction, while, *Citrus aurantium* peel extract revealed improve the morphology of spermatocytes and spermatid appear more regularly and numerous. However, both extracts revealed some histological changes in liver. Moreover, there are no enough documents in the literature about the probable toxic effects of *Punica granatum* and *Citrus aurantium* peel extract. Flora[57] reported that flavonoids are thoughts to have proxidant and antioxidant property on the body.

While the antioxidant protects the tissues and organs, the proxidant damages the tissues and organs. In addition, the toxic effect of *Punica granatum* plant extracts could be related to its alkaloid content.[51] As well, the tannins in pomegranate fruit should be taken into concern for their toxic probable. This may explain some alterations in liver of rabbit treated with *Punica granatum* and *Citrus aurantium* peel extract in the present study. Hence, it can be assumed that the utilization of *Punica granatum* and *Citrus aurantium* is not suggested for a long period due to their negative relation to hepatocytes histological alteration.

Acknowledgement: This work was supported by the national research centre in-house project no. p100702.

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Source of Support: Nil, **Conflict of Interest:** None.