

Protective Role of Polyphenols (Anthocyanin, Gallic Acid) and Blackberry Juice against Acrylamide Reproductive Toxicity in Male Rats

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Abstract

Background: Acrylamide (ACR) is carcinogenic to humans; it is capable of inducing genotoxic, carcinogenic, developmental, and reproductive effects in animals. Blackberries rank highly among fruits with strong antioxidant activities, it is highly concentrated in a vast amount of phytochemicals, most of which are phenolic compounds.

Aim of the study: this study aimed to investigate the antioxidant effect of Blackberry juice (BBJ) as a natural source of polyphenols and either anthocyanin or gallic acid against the harmful effect of acrylamide on reproductive system in male rats. **Methods:** Male albino rats (n=50) averaging 180±5g BW were classified into five equal groups (G; n=10/G). G1 served as untreated control (negative control); G2 (positive control) rats were orally given acrylamide at 50mg/kg BW; G3 rats were given anthocyanin (5mg/kg BW) plus 50 mg/kg BW acrylamide; G4 rats were given 3µg/kg Gallic acid plus 50 mg/kg acrylamide and G5 animals were given 50 mg/kg BW acrylamide plus 1.6g/kg BW of BBJ. Rats were administered their respective doses orally on daily basis for eight weeks. **Results:** Number of sperms, percent motility and plasma testosterone were significantly decreased in acrylamide group, but number of abnormal sperms was significantly increased than in control rats. ACR significantly increased levels of TBARS and NO in testes tissue than control, but it caused significant decreases in the activities of GSH, SOD and TAC in testes tissue than control. ACR also caused significant increase of cytokines (p53, TNF, IL6) than control. Involvement of polyphenols (Anthocyanin, Gallic) along with acrylamide caused improvement in the reproductive functions. Inclusion of blackberry caused significant increases in the number of sperm, motility and plasma testosterone, but decreased number of abnormal sperm compared with anthocyanin and Gallic. Polyphenols (anthocyanin, gallic) and blackberry significantly decreased TBARS, NO and cytokines (p53, TNF and IL6); whereas it increased activities of GSH, SOD and TAC than acrylamide-intoxicated rats. Testicular histological study revealed that ACR showed the maximum affection, but polyphenols and blackberry minimized the deleterious effects of ACR. **Conclusion:** We can conclude that blackberry juice, as a natural source of antioxidant and other nutrients, can alleviate the toxic effects of ACR and enhance the antioxidant defense mechanisms to protect reproductive function surpassing the use of polyphenols (Anthocyanin, Gallic).

Keywords – Acrylamide, Blackberry, Polyphenols, Reproductive Traits, Rats.

I. INTRODUCTION

Acrylamide (ACR) is an organic carcinogenic material in mammals. It is found in starchy foods after treatment at high temperatures (above 120°C) (1, 2) and could also be detected in smog from smoking (3). In addition, people can be exposed to ACR in industrial environment (4). The potential toxicological effects of ACR have been reported in animal

models. Toxicological effects include neurotoxicity, genotoxicity, reproductive toxicity, and embryotoxicity. Glycidamide one of the ACR metabolites, is believed to be the cancer-risk agent in case of the ACR exposure (5). In rats, tumorigenesis occurs in several hormonally regulated tissues (6, 7). Some previous studies showed that ACR is capable of inducing genotoxic, carcinogenic, developmental, and reproductive effects in exposed animals.

Some studies recently have demonstrated that acrylamide-induced cytotoxicity was relevant to oxidative stress (8, 9). The cytotoxic properties of ARC by affecting the cellular redox status might lead to generation of reactive oxygen species (ROS), that may cause cytotoxic and genotoxic effects. Considering this, researchers endeavor to delve some strategies to reduce ARC mediated cytotoxicity (9-11). Berries are highly concentrated in a vast amount of phytochemicals, most of which are phenolic compounds, including flavonoids, condensed and hydrolyzable tannins, stillbenoids, phenolic acids, lignans, triterpenes and sterols. These berry phenols possess strong antioxidant and anti-inflammatory properties, as well as several other anticarcinogenic- related activities that have been thoroughly reviewed elsewhere (12-15).

Blackberries rank highly among fruits with strong antioxidant activities (16, 17). It contains surplus levels of phenolic compounds, mainly gallic and anthocyanins (18, 19), two of the most common phenolic compounds in berries that have been related to antioxidant activity in these fruits (15).

A large number of studies have confirmed that blackberry possesses potent biological activity and may provide health benefits including anti-hyperglycemic, anti-obesity as well as anti-inflammatory effects (20–22). It is noteworthy that food is subjected to digestive conditions within the gastrointestinal tract. During digestion, not only antioxidants, but also many other functional components are immersed in digestive juices. As a consequence, the biological activities of functional components may be altered and some substances may be transformed into other compounds with varied bioactivity and bioavailability (23).

Therefore, the aim of the present study was to investigate the antioxidant effect of Blackberry as a natural source of polyphenols compared with anthocyanin and gallic acid to alleviate the harmful effects of acrylamide on the reproductive system in rats.

II. MATERIALS AND METHODS MATERIALS

All reagents were of analytical grade. Acrylamide (99.9%), anthocyanin and gallic acid were purchased from Sigma Company. Fresh blackberry was obtained from local market, washed, homogenized and its juice was daily prepared for freshness.

The BBJ dose (1.6 g/kg BW) was utilized, as 1kg blackberry contains 3170 mg anthocyanin; so, 1.6 g blackberry contain 5mg anthocyanin (24). Anthocyanin dose was 5 mg /kg BW as found in blackberry. Also, 1kg of

blackberry had approximately 2 mg gallic acid and therefore 1.6 g blackberry contains 3.2 µg gallic acid. (25), so gallic acid dose was 3.2 µg/kg BW. (25) . The tested dose of acrylamide as 1/3 LD50 (26) was 50mg/kg BW for intoxicated rats.

2.1. Experimental animals

Male albino adult rats (n= 50) averaging 180±5g BW were obtained from the animal house of the Medical Research Institute, Alexandria University, Egypt. The local committee approved the design of the experiments and the protocol follows the guidelines of the National Institutes of Health (NIH) (27). Animals received human care, and had adequate stable diet and water *ad libitum*. Animals were acclimatized to the laboratory conditions for two weeks before the commencement of the experiment.

The study was approved by the institutional review board and the ethics committee. The study conformed to the international guidelines on research ethics of animal experimentation. All laboratory biological specimens and hazardous waste were disposed of safely.

2.2. Experimental design

After two weeks of acclimatization, animals were classified into five equal groups (10 rats/ G). G1 animals served as untreated control (negative control); G2 rats were orally given acrylamide dissolved in distilled water at a dose of 50mg/kg BW (positive control); G3 rats were given anthocyanin at a dose of 5 mg/kg BW orally in addition to 50 mg/kg BW acrylamide; G4 rats were given 3µg/kg gallic acid in addition to 50 mg/kg acrylamide and G5 animals were given 50 mg/kg BW acrylamide plus 1.6 g/kg BW of BBJ. Rats were administered their respective doses on daily basis for eight consecutive weeks.

2.3. Body and organs weights

Body weights of the rats were recorded at the beginning, weekly and in the end of the experimental period. Animals were sacrificed by decapitation; testes and epididymis were immediately removed and weighed. Relative organ weights were calculated as g/100g BW. Testes and epididymis were excised immediately.

2.4. Blood sampling

Blood samples were collected from the sacrificed animals in heparinized tubes. Plasma samples were harvested after centrifugation at 4000 rpm for 20 minutes, and then samples were stored at –20°C until used for further analyses

2.5. Sperm parameters

The testes from each rat were carefully exteriorized and one of them was removed together with its epididymis. The spermatozoa were obtained from caudal epididymis. Caudal epididymis sperm density (count), grade of sperm motility and abnormal sperms percent were determined (28). Testosterone was assayed in plasma according to Harding and Velotta (29).

2.6. Antioxidant enzymes activity and free radicals

The activity of superoxide dismutase (SOD) was determined according to the method described by Mishra and Fridovich (30), glutathione (GSH) was determined according to the method of Beutler *et al.* (31), thiobarbituric acid-reactive substances (TBARS) were measured by the method of Tappel and Zalkin (32), total antioxidant capacity (TAC) was assayed by the method of Koracevic *et al.* (33). The level of nitric oxide (NO) was assayed by the method of Montgomery and Dymock (34).

2.7. Plasma Interleukin-6, tumor necrosis factor- α and tumor suppressor gene p53

Rat Interleukin-6 (IL-6) was assayed in plasma by using Enzyme-linked Immunosorbent Assay (ELISA) commercial kit for the *in vitro* quantitative measurement of rat IL-6 (Kamiya Biomedical Co., Gateway Drive, Seattle WA, USA). Tumor necrosis factor- α (TNF- α) was quantitatively determined in plasma samples by using commercial ELISA kits (Abcam co., UK). Also, tumor suppressor gene p53 was assayed by using commercial ELISA kits (Active Motif Co., Palomar Oaks Way, Carlsbad CA, USA).

2.8. Histological study

Testis specimen used for histological study was fixed in neutral formalin for a week at room temperature, dehydrated then cleared in xylene and embedded in paraffin wax. The paraffin sections were cut at 20 microns thickness and stained with Hematoxylin and Eosin for histological examination using the light microscope (35).

2.9. Statistical analysis

All data were expressed as mean $\pm$ SD. Statistical Analyses System (SAS) software program version 9.1 (SAS, 2003) (36) was used for one-way analysis of variance (ANOVA) at $P \leq 0.05$ as the significance level among groups.

Ethical considerations: The study protocol was approved by the institutional review board and the ethics committee of the Institute of Graduated Studies and Researches, Alexandria University, Egypt. The research conformed to the international guidelines on research ethics of animal experimentation. All laboratory biological specimens and hazardous waste were disposed of safely.

III. RESULTS

3.1. Body weight gain and organs relative weights

Table (1) shows a significant ($P < 0.05$) decrease in body weight of the acrylamide- intoxicated rats by 2.2 g of the initial weight. Negative control rat's weight increased by 20.5 g during the experiment; while in the anthocyanin, gallic and blackberry groups the body weight increased by 25.3, 16.1 and 21.3 g, respectively.

Table 1. Changes in body weight gain (g) and relative weight of testes and epididymis (g/100g BW) in the acrylamide- and antioxidants –treated compared with control male rats.

Parameter	Experimental group				
	Control	Acrylamide	Acrylamide + Anthocyanin	Acrylamide + Gallic	Acrylamide + Blackberry
Initial weight (g)	255.0 $\pm$ 7.0 ^a	248.3 $\pm$ 3.6 ^a	252.5 $\pm$ 9.2 ^a	248.3 $\pm$ 10.1 ^a	245.6 $\pm$ 13.3 ^a
Final weight (g)	275.5 $\pm$ 10.4 ^a	246.1 $\pm$ 4.7 ^b	277.8 $\pm$ 4.2 ^a	264.4 $\pm$ 8.5 ^b	266.9 $\pm$ 7.9 ^b
Body weight gain (g)	20.5 $\pm$ 11.5 ^b	-2.2 $\pm$ 2.3 ^d	25.3 $\pm$ 9.5 ^a	16.1 $\pm$ 8.8 ^c	21.3 $\pm$ 9.8 ^b
Testes (g/100g BW)	1.01 $\pm$ 0.07 ^b	1.17 $\pm$ 0.03 ^a	1.02 $\pm$ 0.03 ^b	1.12 $\pm$ 0.03 ^{ab}	1.08 $\pm$ 0.04 ^{ab}
Epd (g/100 g BW)	1.3 $\pm$ 0.05 ^a	1.3 $\pm$ 0.04 ^a	1.4 $\pm$ 0.1 ^a	1.4 $\pm$ 0.03 ^a	1.4 $\pm$ 0.07 ^a

Results are expressed as mean $\pm$ SE; Means with different

superscript letters in the same row imply significant

differences at $P \leq 0.05$.

3.2. Reproductive function

Reproductive functions are summarized in table 2. It has been noticed that number of sperms significantly ($P < 0.05$) decreased in acrylamide-given rats ($13.0 \times 10^6/\text{ml}$) compared with control rats ($79.0 \times 10^6/\text{ml}$). Also, percent of abnormal sperms significantly ($P < 0.05$) increased in acrylamide-intoxicated (54.0%) than in control (11.3%) rats. Progressive motility of the sperm appreciably declined in acrylamide (9.0%) than in control animals (75.0%).

Inclusion of polyphenols (anthocyanin, gallic) along with acrylamide improved reproductive functions in male rats. Among treatments, blackberry increased ($P < 0.05$) sperm concentration ($57.7 \times 10^6/\text{ml}$) than anthocyanin ($18.3 \times 10^6/\text{ml}$) and gallic ($39.0 \times 10^6/\text{ml}$). Likewise, blackberry caused significant ($P < 0.05$) decrease in the percentage of abnormal sperm (19.0%) followed by gallic (25.0%) and anthocyanine (37.0%). Furthermore, blackberry significantly increased percentage of sperm motility (38.3%) compared with gallic (30.0%) and anthocyanin (27.7%).

Table 2. Changes in sperm morphology of rats in the acrylamide-and antioxidants-treated compared with control male rats.

Parameter	Experimental groups (mean $\pm$ SD)				
	Control	Acrylamide	Acrylamide+ Anthocyanin	Acrylamide+ Gallic	Acrylamide +Black berry
Concentration (Million/ml)	79.0 ± 0.6^a	13.0 ± 0.6^d	18.3 ± 0.9^d	39.0 ± 2.3^c	57.7 ± 3.4^b
Abnormal (%)	11.3 ± 0.3^d	54.0 ± 3.2^a	37.0 ± 3.5^b	25.0 ± 2.3^c	19.0 ± 0.6^c
Motility (%)	75.3 ± 5.2^a	9.0 ± 0.6^d	22.7 ± 1.5^c	30.0 ± 0.6^c	38.3 ± 2.1^b

Results are expressed as mean $\pm$ SE; Means with different superscript letters in the same row imply significant differences at $P \leq 0.05$.

3.3. Plasma testosterone

Figure 1 illustrates that ACR significantly ($P < 0.05$) reduced plasma testosterone concentration (0.96 ng/ml) reaching about 20% of that found in control animals (4.7 ng/

ml). On the other hand, it has been found that gallic and blackberry equally recovered plasma testosterone (3.0 ng/ml), however the anthocyanin was less effective in recovering the normal testosterone level (1.95 ng/ml).

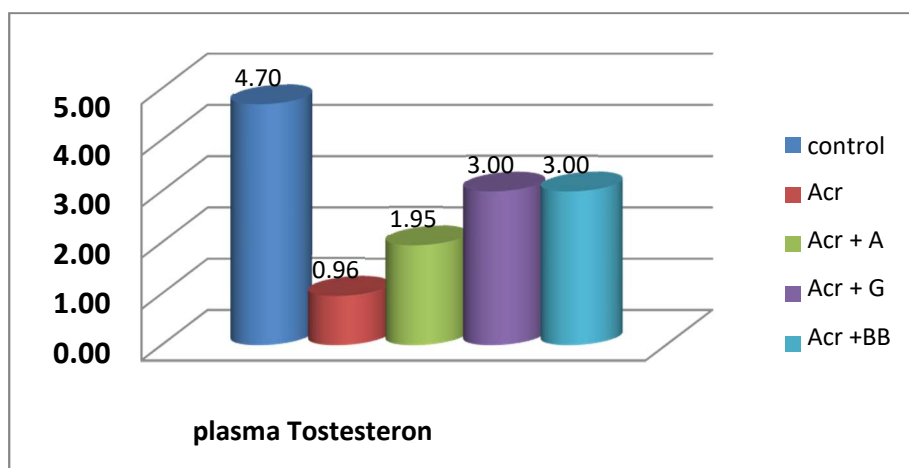


Fig.1 Effect of acrylamide and antioxidants on Plasma testosterone of male rats.

3.4. Antioxidants

Table 3 exhibits that ACR significantly ($P < 0.05$)

increased level of TBARS (193.6 nmol/g) and NO (55.7 $\mu\text{mol/g}$) in the testicular tissues compared with their

counterparts in control rats (28.4 nmol/g and 33.9 μ mol/g, respectively). On the contrary, ACR caused significant ($P < 0.05$) reductions in the activities of GSH (5.6 μ mol/g), SOD (397.5 μ mol/g) and TAC (0.4 mmol/g) in the testicular tissues compared with their counterparts in control rats (6.7 μ mol/g, 612.3 μ mol/g and 1.4 mmol/g, respectively). While inclusion of polyphenols (anthocyanin, gallic) and blackberry along with acrylamide significantly ($P < 0.05$) decreased TBARS (29.6, 41.9 and 27.2 nmol/g in

anthocyanin, gallic and blackberry, respectively) and NO (37.7, 43.1 and 39.3 μ mol/g in anthocyanin, gallic and blackberry, respectively). Contrariwise, polyphenols (anthocyanin, gallic) and blackberry significantly ($P < 0.05$) raised the activities of GSH (6.8, 5.7 and 6.9 μ mol/g in anthocyanin, gallic and blackberry, respectively); SOD (459.1, 483.9 and 602.0 μ mol/g in anthocyanin, gallic and blackberry, respectively) and TAC (1.43, 1.43 and 1.5 mmol/g in anthocyanin, gallic and blackberry, respectively).

Table3. Levels and activities of antioxidants compounds and enzymes in the testicular tissues in the acrylamide-and antioxidants–treated compared with control male rats.

Parameter	Experimental group				
	Control	Acrylamide	Acrylamide+ Anthothyanin	Acrylamide+ Gallic	Acrylamide Black berry
TBARS nmol/g tissue	28.4 $\pm$ 1.2 ^c	193.6 $\pm$ 4.9 ^a	29.6 $\pm$ 1.4 ^c	41.9 $\pm$ 1.8 ^b	27.2 $\pm$ 0.6 ^c
NO μ mol/g tissue	33.9 $\pm$ 0.7 ^d	55.7 $\pm$ 0.5 ^a	37.7 $\pm$ 0.9 ^c	43.1 $\pm$ 1.2 ^b	39.3 $\pm$ 0.4 ^c
GSH μ mol/g tissue	6.7 $\pm$ 0.01 ^a	5.6 $\pm$ 0.04 ^b	6.8 $\pm$ 0.12 ^a	5.7 $\pm$ 0.2 ^b	6.9 $\pm$ 0.1 ^a
SOD μ mol/g tissue	612.3 $\pm$ 7.01 ^a	397.5 $\pm$ 8.5 ^c	459.1 $\pm$ 8.3 ^b	483.9 $\pm$ 19.0 ^b	602.0 $\pm$ 9.4 ^a
TAC mmol/g tissue	1.4 $\pm$ 0.0 ^a	0.4 $\pm$ 0.02 ^c	1.43 $\pm$ 0.01 ^b	1.43 $\pm$ 0.01 ^b	1.5 $\pm$ 0.01 ^{ab}

Results are expressed as mean $\pm$ SE; Means with different superscript letters in the same row imply significant differences at $P \leq 0.05$.

3.5. Cytokines

ACR caused significant ($P < 0.05$) increases in cytokines [p53 (25.5 pg/ml); TNF (120.5 pg/ml) and IL6 (209.3 pg/ml)] compared with control animals (6.2, 41.5 and 10.5 pg/ml for p53, TNF and IL6, respectively). The increases approached 311,190 and 108%, respectively over the control. While involvement of anthocyanin, gallic and blackberry

significantly decreased ($P < 0.05$) p53 (10.7, 14.0 and 9.4 pg/ml, respectively) compared with the ACR-given rats by 58%, 45.1% and 63.1%, respectively. It also decreased ($P < 0.05$) TNF (71.0, 70.0 and 50.0 pg/ml) and IL6 (124.5, 133.5 and 91.0pg/ml) compared with its counterparts in ACR-given animals by a range of reduction between 36.2% and 56.5%.

Table4. Levels of plasma cytokines (P53, TFN, IL) in the acrylamide- and antioxidants–treated compared with control male rats.

Parameter	Experimental group				
	Control	Acrylamide	Acrylamide + Anthothyanin	Acrylamide + Gallic	Acrylamide + Black berry
P53(pg/ml)	6.2 $\pm$ 0.2 ^d	25.5 $\pm$ 0.7 ^a	10.7 $\pm$ 0.3 ^c	14.0 $\pm$ 0.4 ^b	9.4 $\pm$ 0.6 ^c
TFN(pg/ml)	41.5 $\pm$ 1.4 ^d	120.5 $\pm$ 4.9 ^a	71.0 $\pm$ 1.2 ^b	70.0 $\pm$ 2.3 ^b	50.0 $\pm$ 0.6 ^c
IL6(pg/ml)	100.5 $\pm$ 1.6 ^c	209.3 $\pm$ 2.7 ^a	124.5 $\pm$ 4.9 ^b	133.5 $\pm$ 3.8 ^b	91.0 $\pm$ 5.2 ^c

Results are expressed as mean $\pm$ SE; Means with different superscript letters in the same row imply significant differences at $P \leq 0.05$.

3.6. Testicular histopathology

Figure 2a illustrates great damages to the spermatogenic cells (S) in the ACR- intoxicated rats. Complete destruction of spermatogenic cells accompanied with detachment from their basement membranes and empty lumens from mature

sperms (arrow). Invasion of blood vessels (BV) in-between seminiferous tubules (ST) was strikingly unusual. Administration of the antioxidants (Fig.2 b,c,d) didn't completely ameliorate the damaging effects of acrylamide on the testicular architecture.

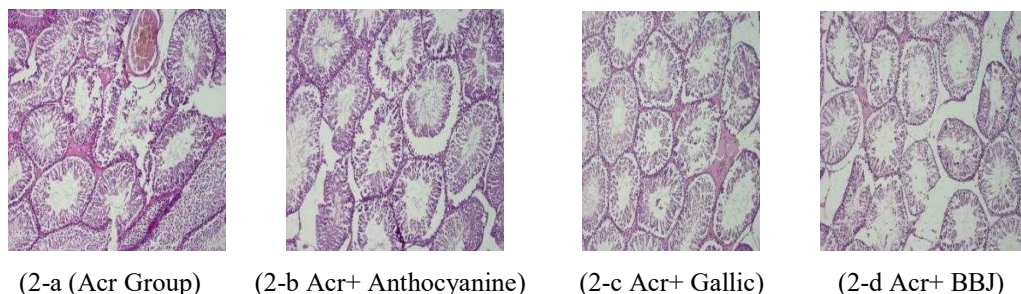


Figure 2 (a-d):Light photomicrographs of rat testes showing sections of seminiferous tubules (ST) of experimental groups illustrating different levels of destruction in the form of wide spacing, with areas of lymphatic extravasations in between S.T (Mic. Mag X100)

IV. DISCUSSION

Blackberries are a rich source of anthocyanin and other phenolic compounds, and they have great health-promoting functions, such as anticancer properties, neuro-protective effects, antioxidant properties and alleviation of high blood pressure (37-40). In the current study, it is evident that phenolic compounds (anthocyanin, Gallic) and blackberry positively recovered the reduction in the body weight loss occurred due to the ACR. Yang et al suggested that ACR administration could decrease body weight of animals, this possibly because of the decreased food and water intakes (41). The detrimental effect that has been occurred in the ACR-intoxicated rat's testicles showing deterioration of seminiferous tubules, lower sperm count and sharp decline of testosterone would be clearly explained from the concept that the acrylamide ability to pass through the blood-testis barrier and disrupt the growth and development of spermatogenesis and testosterone production. This finding was consistent with what others found earlier in rats (41-43).

Ma et al. also found that sperm abnormalities observed in the ACR-treated animals revealed lacking in hook, banana-like head, big head, amorphous head, twin head, with head abnormalities, twin-tail, folded on tail and defective tail (44). It has been suggested that sperm quality status could reflect reproductive impairment during the early stage of development (45). Moreover, previous researchers found decreases of sperm motility and sperm survival rate due to acrylamide provision. The interferences that caused by acrylamide on the sperm cell metabolism could be a clear

interpretation for the sperm motility reduction (44). Furthermore, it has been reported that developmental toxicity may be associated with the formation of protein adduct. This would be explained from the point that the main metabolite of acrylamide is glycidamide which owe the ability to bind to the sperm protamines, leading to dominant lethal and sperm distortion. Both ACR and its metabolite; glycidamide could combine with dynein and hence affect impede sperm motor ability (46). Blackberry enhanced sperm motility and viability; recovering, in part, its vitality which was deteriorated by the provision of acrylamide. Effect of blackberry juice against the toxic effect of Chlorpyrifos (CPF) was investigated by Hamza who found that blackberry juice alone showed no changes on sperm concentration and motility, while reduced non-viable and abnormal spermatozoa. However, treatment with a combination of blackberry juice with CPF resulted in not only alleviation to the declines in sperm concentration and motility but it also increased percent of sperm viability and reduced abnormalities. Same author suggested that blackberry juice minimized the reproductive toxicity of CPF. Moreover, blackberry juice significantly increased testosterone and mitigated the negative effects of CPF (47).

Antioxidant enzymes such as SOD and CAT can protect cellular compounds against damage induced by free radicals. Therefore, the activities of these enzymes have been used to assess oxidative stress in cells (48, 49). In the present study, it was found that ACR increased NO and TBARS levels, but decreased activities of GSH, SOD and TAC. Presence of polyphenols (anthocyanin and Gallic) or blackberry along

with ACR decreased NO and TBARS levels significantly. In consistent with the current results, Xuenan et al also found that ACR induced significant decreases in the activities of SOD and CAT compared with the non-treated animals. Likewise, SOD and CAT activities significantly increased in the animals administered with anthocyanin compared with the ACR-provided animals (50, 51). On the other hand, Hassan and Yousef found that blueberry juice reduced sodium fluoride-induced TBARS and NO. Moreover, BBJ ameliorated sodium fluoride-induced reductions in the activities of SOD, CAT and GSH. They suggested that BBJ owes a protective effect against sodium fluoride- induced hepatotoxicity by antagonizing the free radicals generation and enhancement of the antioxidant defense mechanisms (52).

In the present study, ACR caused significant increases in the studied cytokines (p53, TNF and IL6), which are in agreement with other study (53).

AL Karim et al. also found that ACR administered with the ordinary rat's food induced adverse effects on male reproductive system as reflected in the reductions of absolute weights of testes and seminal vesicles (53). They also found that seminal vesicles and testes appeared smaller in size. Moreover, the seminal vesicles were less lobulated and the testes showed congested blood vessels compared to the control group. After 90 days of feeding of ACR, all previous changes were evidently observed. Some testes showed atrophy and separation of seminiferous tubules and interstitial edema in the testicular parenchyma.

V. CONCLUSION

From the present finding, it can be concluded that blackberry juice, as a natural source of antioxidants and other nutrients, can alleviate the toxic effects of ACR. Furthermore, it enhances the antioxidant defense mechanism leading to the protection of the reproductive functions at a rate surpassing the use of either regular polyphenols (Anthocyanin and Gallic) separately.

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