Supplementation of *Nigella sativa* fixed and essential oil mediates potassium bromate induced oxidative stress and multiple organ toxicity

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Abstract: The plants and their functional ingredients hold potential to cure various maladies and number of plants hold therapeutic potential. The present research was designed study the health promoting potential of black cumin (*Nigella sativa*) fixed oil (BCFO) and essential oil (BCEO) against oxidative stress with special reference to multiple organ toxicity. For the purpose, thirty rats (Strain: Sprague Dawley) were procured and divided into three groups (10 rats/group). The groups were fed on their respective diets i.e. D_1 (control), D_2 (BCFO @ 4.0%) and D_3 (BCEO @ 0.30%) for a period of 56 days. Mild oxidative stress was induced with the help of potassium bromate injection @ 45 mg/Kg body weight. Furthermore, the levels of cardiac and liver enzymes were assayed. The results indicated that oxidative stress increased the activities of cardiac and liver enzymes. However, supplementation of BCFO and BCEO was effective in reducing the abnormal values of enzymes. Elevated levels of lactate dehydrogenase (LDH), CPK and CPK-MB were reduced from 456 to 231, 176 to 122 and 45 to 36mg/dL, respectively. Similarly, liver enzymes were also reduced. However, the results revealed that BCEO supplementation @ 0.30% is more effectual in ameliorating the multiple organ toxicity in oxidative stressed animal modelling. In the nutshell, it can be assumed that black cumin essential oil is more effective in reducing the extent of potassium bromate induced multiple organ toxicity (cardiac and liver enzymes imbalance) that will ultimately helpful in reducing the extent of myocardial and liver necrosis.

Keywords: Plants, nutritional requirements, oxidative stress, cardiac, liver enzymes.

INTRODUCTION

In counties like Pakistan, plants are used in traditional medicines to treat and cure various maladies. The health promoting potential associated with their consumption is attributed to presence of bioactive molecules. In this regard, natural products hold promising position to safeguard humans from deleterious effects of free radicals (Butt *et al*., 2009). These free radicals are produced during the oxidation process necessary for energy metabolism. Moreover, pollution, pesticide residues, heavy metal toxicity, ingestion of toxicants and direct exposure to ultraviolet radiations are other causes of their production (Valko *et al*., 2007). The antioxidants present in the body act as first line of defence to scavenge them but imbalance balance between their production and antioxidants results in oxidative stress (Ramaa *et al*., 2006). Free radicals being reactive are very destructive in nature and cause damage to macromolecules. In this regard, oxidation of low-density lipoprotein (LDL) is major causative agent in atherosclerosis (Butt *et al*., 2009). Overall, oxidative stress results in DNA damage, cardiovascular & neurodegenerative disorders and cancers (Gackowski *et al*., 2008). The levels of some indigenous enzymes present in human body are also altered under these pathological states (Clarkson *et al*., 1995). However, extra antioxidant supplementation through diets can be helpful in bolster defence mechanisms (Sánchez-Reus *et al*., 2007; Migliore *et al*., 2008).

Globally, the consumer's interests faltered towards nature and numerous research studies were designed to expedite the role of natural antioxidants. In this regard, supplementation of antioxidant through dietary modules might be helpful in prevention of oxidative stress and allied ailments. The phytochemicals present in plants including tocopherols, thymoquonne, flavonoids, and phenolic compounds are of significance important. These bioactive molecules possess antioxidant activity that is proven through various *in vitro* and *in vivo* modules (Mustafa *et al*., 2010). Some chemical compounds belonging to families of flavonoids, terpenoids, and carotenoids are also important in this regard (Van Acker *et al*., 2000).

The induction of oxidative stress in Sprague Dawley rats is characterized with the disparity in cardiac and liver

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functionality. There are several plants that can be exploited for the extraction of natural products (Ghasemi *et al*., 2009; Ebrahimzadeh *et al*., 2010). The black cumin fixed oil (BCFO) contains appreciable quantities of tocopherols and phytosterols. In contrary, the black cumin essential oil (BCEO) obtained through the process of distillation contains antioxidant & alkaloids. These phytochemicals rich fractions can be utilized to ameliorate multiple organ toxicity associated with oxidative stress i.e. BCFO and BCEO. The essential oil contains volatiles constituents, whilst fixed oil is lipid fraction containing fatty acids and fat-soluble vitamins (tocopherols) and phytosterols. In the present study, black cumin fixed and essential oils (BCFO & BCEO) that are rich source of dietary phytochemicals were explored (Sultan *et al*., 2009a). The levels of enzymes as indices of multiple organ toxicity were estimated and effects of BCFO & BCEO supplementation were studies. The outcomes of present project would be helpful for designing such studies in human subjects in order to prevent the onset of degenerative disorders.

MATERIALS AND METHODS

The black cumin (indigenous variety) seeds were gifted from government research institute situated in Chakwal (Barani Agricultural Research Institute). Chemical Reagents (analytical & HPLC grade) and standards were purchased from Sigma-Aldrich Tokyo, Japan and Merck KGaA, Darmstadt, Germany. National Institute of Health (NIH) Islamabad, Pakistan provided infectious free Sprague dawley rats for the research purpose as per instructions of “Animal Care Committee, NIFSAT-Faisalabad Pakistan”.

Extraction of black cumin fixed and essential oils

The black cumin fixed oil was extracted using the procedures outlines in AOAC (1998). Briefly, the seeds were slurred with the organic solvent (n-Hexane) in the ratio of 1:6. The process was carried out using soxhtec apparatus and extra amounts of solvent were separated using Rotary Evaporator. The results pertaining to the nutritional composition of BCFO are already published in Pakistan Journal of Botany (Sultan *et al*., 2009a). In comparison, the black cumin essential oil was extracted using locally assembled hydro-distillation apparatus following the protocols of Kanter *et al*. (2003) and Sultan *et al*. (2009a).

Induction of oxidative stress

Oxidative stress was induced in rats with the peritoneal injection of potassium bromate @ 45mg/kg body weight. The potassium bromate was dissolved in citrate buffer (0.05M) and pH was adjusted at 4.5. The rats were further fed on oxidized corn oil (POV: 120 meq/g) to produce chronic oxidative stress. The three different experimental diets were prepared i.e. control, diet with 4.0% BCFO, and 0.03% BCEO. Later, the effects of these tests diets were studied through evaluation of indices of multiple organ toxicity (cardiac and liver enzymes).

Housing of rats

Thirty Sprague Dawley rats were used in the present study and divided into 3 groups (10 each). The rats were fed on basal diet for one week duration of acclimatize them with the environments. Later, experimental diets (table 1) were fed for the duration of 56 days. The animals were maintained according to standard guidelines of Animal Institute of Nutrition (AIN), USA i.e. temperature 23±2ºC, relative humidity 55±5%, and 12-hr light-dark cycle. The analytical procedures carried out include some parameters measured daily (feed and water intake) and body weight was measure on weekly basis. At 28 and 56 days of feeding trials, five rats from each group were decapitated and blood was collected through neck and cardiac puncture (Uchida *et al*., 2001). The blood samples collected were analyzed for further analysis that is mentioned herein.

Table 1: Composition of control and experimental diets

Diet Constituents	D_1	D_2	D_3
Casein (g)	20.0	20.0	20.0
Corn starch (g)	55.0	55.0	55.0
Cellulose	10.0	10.0	10.0
Corn oil (g)	10.0	6.0	10.0
Black cumin fixed oil	-	4.0	-
Black cumin essential oil	-	-	0.3
Mineral mixture (g)	4.0	4.0	4.0
Vitamin mixture (g)	1.0	1.0	1.0
Total diet weight (g)	100	100	100

D_1 = Control diet; D_2 = Black cumin seed fixed oil; D_3 = Black cumin seed essential oil.

Indices of multiple organ toxicity

The biomarkers of multiple organ toxicity estimated in the present research include aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). The commercials kits were purchased from ChronoLab, Switzerland that employs enzymatic methods. These tests were carried out to check the membrane damage. Moreover, bilirubin-conjugated proteins contents were also assayed using commercial kits.

Indices of kidney and digestive functionality

Urea, creatinine and uric acid were assayed using commercial kits to determine the influence of diets in ameliorating kidney and digestive system functionally.

Biomarkers of myocardial necrosis

The biomarkers of myocardial necrosis estimated in the present research include lactose dehydrogenase (LDH), Creatine phosphokinase (CPK), and creatine phosphokinase-MB (CK-MB) following the protocols described by Kairisto *et al*. (1993). Briefly, activities of LDH, CPK and (CK-MB) were determined using commercially available kits from Biocon® (Germany), Cayman Chemicals, and Stanbio Laboratory (Boerne, TX), respectively.

STATISTICAL ANALYSIS

Statistical package i.e. Cohort V-6.1 (Co-Stat Statistical Software, 2003) was used for data analysis. Briefly, values presented in Tables are means ± standard deviation. In order to check the level of significance, analysis of variance (ANOVA) technique was applied. The diets (factor A), intervals (factor B) and their interaction (A × B) were used as source of variations. Duncan's multiple range test (DMRt) further clarified the effects of diets in a comprehensive manner.

RESULTS

Before we describe the results pertaining to present research, it should be kept in mind that safety assessment of black cumin fixed and essential oil has been already published carried by same group (Sultan *et al*., 2009b). Their results were conclusive that BCFO and BCEO @ of 4.0 and 0.3% are safe for humans. In their research, the levels of cardiac and liver enzymes remained in the normal ranges (Morita *et al*., 2008). The present research was a step ahead in the direction and induction of oxidative stress by means of potassium bromate injection was carried out.

Markers of liver damage

Overall, liver enzymes i.e. AST, ALT and ALP and bilirubin-conjugated proteins (direct and indirect bilirubin) are of cardinal importance. The results regarding ALT indicated that activities of this enzyme increased from 108.54±6.96 to 152.27±9.47IU/L in control groups. However, supplementation of BCFO and BCEO attenuated the adverse consequences of oxidative stress; levels of ALT decreased from 112.58±7.25 to 70.22±4.12 and 107.80±4.26 to 77.04±3.30IU/L, respectively (table 2). Likewise, AST decreased in test diets from 172.97±11.14 to 156.88±9.21 and 170.35±6.73 to 135.64±5.80IU/L, respectively, while it increased from 178.05±11.41 to 242.08±15.06IU/L in control diets (table 3). Similar pattern has been observed for ALP; increased from 388.86±24.93 to 689.95±42.92IU/L in control diets. The test diets containing BCFO and BCEO improved the conditions as indicated from decreasing trends for ALP from 412.95±26.60 to 292.00±17.15 and 403.57±15.94 to 232.02±9.93IU/L, respectively (table 4).

Table 2: Level of ALT (IU/L) in oxidative stressed rats

Diets	Study intervals (Days)		
	0	28	56
D_1	108.54±6.96	125.90±9.08	152.27±9.47
D_2	112.58±7.25	96.25±4.49	70.22±4.12
D_3	107.80±4.26	86.27±4.13	77.04±3.30

ALT = Alanine aminotransferase:
Means carrying same letters in a column/row do not differ significantly.
D_1 = Control diet; D_2 = Black cumin seed fixed oil (4%); D_3 = Black cumin seed essential oil.

Table 3: Level of AST (IU/L) in oxidative stressed rats

Diets	Study intervals (Days)		
	0	28	56
D_1	178.05±11.41	211.65±15.26	242.08±15.06
D_2	172.97±11.14	162.22±7.57	156.88±9.21
D_3	170.35±6.73	156.34±7.48	135.64±5.80

AST = Aspartate aminotransferase
Means carrying same letters in a column/row do not differ significantly.
D_1 = Control diet; D_2 = Black cumin seed fixed oil; D_3 = Black cumin seed essential oil.

Table 4: Level of ALP (IU/L) in oxidative stressed rats

Diets	Study intervals (Days)		
	0	28	56
D_1	388.86±24.93	475.74±34.31	689.95±42.92
D_2	412.95±26.60	357.17±16.68	292.00±17.15
D_3	403.57±15.94	329.45±15.77	232.02±9.93

ALP: Alkaline phosphatase
Means carrying same letters in a column/row do not differ significantly
D_1 = Control diet; D_2 = Black cumin seed fixed oil; D_3 = Black cumin seed essential oil

Bilirubin proteins (total bilirubin, direct bilirubin (mg/dL) and indirect bilirubin) were significantly affected by the diets and remained significant for study intervals and their interaction (table 5). Mean for total bilirubins explicated increasing tendency in control diets i.e. 0.92±0.048 to 1.05±0.027mg/dL. Similar pattern has been observed for direct and indirect bilirubin proteins. The test diet containing BCFO and BCEO owing to the rich phytochemistry reduced the secretions of bilirubins that indicate the normal liver functionality. However, effects were more pronounced for indirect bilirubins; BCFO and BCEO decreased from 0.74±0.040 to 0.56±0.042 and 0.78±0.066 to 0.66±0.039mg/dL, respectively.

Indices of kidney and digestive functionality

Indices of kidney function include urea and creatinine while uric acid is indicator for proper functionality of digestive tract. In the present research investigation, it was concluded from the mean that diets showed

Table 5: Levels of bilirubin proteins (mg/dL) in oxidative stressed rats

Parameters	Diets	Study intervals (Days)		
		0	28	56
Total bilirubin	D_1	0.92±0.048	1.05±0.021	1.05±0.027
	D_2	0.92±0.023	0.79±0.037	0.79±0.027
	D_3	0.99±0.039	0.83±0.026	0.85±0.036
Conjugated bilirubin	D_1	0.16±0.010	0.18±0.015	0.25±0.022
	D_2	0.18±0.010	0.16±0.015	0.23±0.017
	D_3	0.21±0.018	0.20±0.017	0.19±0.011
Un-conjugated bilirubin	D_1	0.76±0.047	0.87±0.074	0.80±0.071
	D_2	0.74±0.040	0.63±0.058	0.56±0.042
	D_3	0.78±0.066	0.63±0.054	0.66±0.039

Means carrying same letters in a column/row do not differ significantly
D_1 = Control diet; D_2 = Black cumin seed fixed oil; D_3 = Black cumin seed essential oil

Table 6: Indices of kidney and digestive functionality (mg/dL) in oxidative stressed rats

Parameters	Diets	Study intervals (Days)		
		0	28	56
Urea	D_1	32.47±2.082	33.22±2.396	36.93±2.297
	D_2	33.14±2.135	29.01±1.354	28.08±1.649
	D_3	32.89±1.299	29.80±1.426	27.29±1.167
Creatinine	D_1	0.86±0.053	0.95±0.081	1.16±0.103
	D_2	0.79±0.042	0.89±0.082	0.90±0.067
	D_3	0.78±0.066	0.82±0.070	0.90±0.054
Uric acid	D_1	6.90±0.429	5.90±0.500	5.47±0.487
	D_2	6.95±0.372	6.22±0.572	5.90±0.438
	D_3	6.72±0.566	6.40±0.545	6.10±0.364

Means carrying same letters in a column/row do not differ significantly
D_1 = Control diet; D_2 = Black cumin seed fixed oil; D_3 = Black cumin seed essential oil

differential impact on all parameters. Means for urea contents varied from 27.29±1.167 to 36.93±2.297mg/dL (table 6). The control diet increased urea contents from 32.47±2.082 to 36.93±2.297mg/dL. Urea contents decreased significantly from 0 days values of 33.14±2.135 and 32.89±1.299mg/dL to 28.08±1.649 and 27.29±1.167mg/dL, respectively in experimental diet groups i.e. BCFO and BCEO.

In contrary, means for creatinine followed the increasing pattern in all diet groups, however, the increase was more pronounced in control groups (table 6). Additionally, decreasing tendency in the level of uric acid was observed and decrease was more promising in group of rats fed on control diets.

Markers of myocardial necrosis

The significant variations among the mean activities for LDH were observed; maximum in control group i.e. 590.00±37.82, 620.00±44.71 and 691.41±43.01mg/dL at 0, 28 and 56 days of study, respectively (table 7). The higher values indicate myocardial necrosis and test diets (BCFO & BCEO) attenuated the conditions and decreased activities from baseline values of 560.15±36.09 and 582.04±22.99 to 391.05±22.97 and 340.28±14.56mg/dL, respectively, at the end of 56 days feeding trials. In contrary to LDH, tests diets maintained the level of CPK during whole study duration. However, increasing tendency was observed in group of rats fed on control diet i.e. 255.60±13.31 to 349.27±9.04mg/dL (table 8).

Table 7: Levels of LDH (mg/dL) in oxidative stressed rats.

Diets	Study intervals (Days)		
	0	28	56
D_1	590.00±37.82	620.00±44.71	691.41±43.01
D_2	560.15±36.09	462.25±21.58	391.05±22.97
D_3	582.04±22.99	469.17±22.45	340.28±14.56

Means carrying same letters in a column/row do not differ significantly
D_1 = Control diet; D_2 = Black cumin seed fixed oil; D_3 = Black cumin seed essential oil

Different studies pertaining to markers of myocardial necrosis presented the evidences that CK-MB can used as efficient diagnostic tool along with some other parameters. In the present study, level of CK-MB was recorded higher at baseline (0 days) that can be used as marker of myocardial necrosis. The control or placebo group showed escalating tendency and activities of CK-MB increased nearly two fold from 26.34±1.69 to 50.50±3.14mg/dL (table 9). In contrary, black cumin fixed oils containing diet portrayed increasing trend but at slower rate i.e. 20.91±1.35 and 26.89±1.58mg/dL at 0 and 56 days of study, respectively. Black cumin essential oil (BCEO) resulted in marked decrease from 25.68±1.10 to 16.91±0.67mg/dL during 56 days feeding trial. The results elaborated that essential oil provided protective shield against free radical damage and resulted in reduced extent of myocardial necrosis.

Table 8: Levels of CPK (mg/dL) in oxidative stressed rats

Diets	Study intervals (Days)		
	0	28	56
D_1	255.60±13.31	312.90±6.26	349.27±9.04
D_2	256.23±6.53	260.56±12.17	268.40±9.10
D_3	258.42±10.21	265.93±8.37	266.51±11.40

Means carrying same letters in a column/row do not differ significantly
D_1 = Control diet; D_2 = Black cumin seed fixed oil; D_3 = Black cumin seed essential oil

Table 9: Levels of CK-MB (mg/dL) in oxidative stressed rats

Diets	Study intervals (Days)		
	0	28	56
D_1	26.34±1.69	32.24±2.32	50.50±3.14
D_2	20.91±1.35	21.17±0.99	26.89±1.58
D_3	25.68±1.10	24.82±1.19	16.91±0.67

Means carrying same letters in a column/row do not differ significantly
D_1 = Control diet; D_2 = Black cumin seed fixed oil; D_3 = Black cumin seed essential oil

DISCUSSION

The diet and health linkages are well defined and many research studies carried out in animal and humans provided evidences that dietary control is best strategy in preventing many ailments e.g. cardiovascular disorders and diabetes mellitus (Butt and Sultan, 2010). The rich phytochemistry of plants has been employed since long to prevent and cure various maladies. Such strategies resulted in coinage of terms like functional and nutraceuticals foods. These strategies are found more effective in reducing the risk of diabetes mellitus and cardiovascular disorders. The mode of action of these foods involve multidirectional pathways, however, safety assessment through animal modelling is essential for recommending their further use. Black cumin is famous in Asian countries owing to its potential role in prevention of ailments also supported from scientific interventions claiming its potential use as a functional food. The health benefits of black cumin seeds are attributed to bioactive molecules concentrated in it fixed and essential oils. In this regard, Sultan *et al*. (2009b) carried out the safety assessment of black cumin fixed and essential oil (BCFO & BCEO) and reported that both fractions are safe for human consumption. In another study, same fractions were effective in reducing the extent of myocardial necrosis in diabetic rats (Sultan *et al*., 2009c). In the present project, further efforts were made to study the effect of BCFO and BCEO on cardiac and liver enzymes in oxidative stressed Sprague dawley rats.

Oxidative stress was induced with the help of intraperitoneal injection of potassium bromate @ 45mg/kg body weight. The groups of rats were further given oxidized corn oil to keep free radical production going (Khan *et al*., 2003). Our findings concluded that the oxidative stress was influential factor in enhancing the values of cardiac and liver enzymes beyond their normal ranges. The result of such outburst in the activities could further lead to damage to endothelial membranes. The observed values were far higher than reported by Morita *et al*. (2008) and Chengelis *et al*. (2008) for normal rats. The justification for this abrupt increase includes free radical damage to heart and liver tissues resulting in enhanced secretions. The normal ranges for ALP, ALT and AST are 139 to 260, 33 to 81 and 88 to 162 U/L, respectively (Petterino *et al*., 2003). However, the level of these enzymes crossed the normal ranges in 56 days feeding trail. The abnormal increase in their levels was attenuated by the experimental diets containing black cumin fixed & essential oil.

In the past, potassium bromate was used as additive in flour industry but in 1992, it was banned due to its role in free radical production in human/animal subjects. It has dual mechanism of action as it produces free radicals as well as damage membranous structures that covers organs like heart and liver. The treatment of potassium bromate resulted in higher production of free radicals that resulted in marked increase in free radicals induced enzymes activities (Umemura *et al*., 1995). The uncontrolled production could possibly be lead to carcinogenesis (Kurokawa *et al*., 1986; Sai *et al*., 1994). In the present project, free radical production was enhanced with the help of feeding rancid corn oil (Daba *et al*., 1998). The higher production of free radicals resulted in higher AST, ALT and ALP activities. Moreover, bilirubin proteins contents were also observed higher in control group as compared to lower in black cumin fixed and essential oils groups. The improvements in the said parameters were possibly due to presence of antioxidant components in BCFO and BCEO. The other reasons include improvement in antioxidant status of the body influenced by experimental diets. Improvement in antioxidant status of the body certainly assures the normal functioning of the human body (Kökdil1 *et al*., 2006). The rich phytochemistry of black cumin fixed and essential oils includes presence of thymoquinone & tocopherols in BCFO and thymoquinone, carvacrol, thymol, cymene, t-anethole, 4-terpineol in BCEO (Clarkson *et al*., 1995, Wajs *et al*., 2006). Antioxidant potential of experimental diets is certainly due to these functional ingredients. These findings are in agreement with Kanter *et al*. (2003), Zaoui *et al*. (2002) and Meral *et al*. (2001). Similar study conducted by Shinde and Goyal (2003) elucidated that black cumin and its components improve antioxidant potential of body in diabetic rats. Their findings are in accordance with our that supplementation of black cumin fixed and essential oil resulted in significant reduction in the levels of cardiac & liver enzymes and indices of renal and digestive functionality (Sahin *et al*., 2007).

CONCLUSION

Conclusive approach drawn from this part of efficacy study is that black cumin fixed and essential oils hold effectiveness in attenuating the elevated levels of cardiac and liver enzymes owing to oxidative stress induced by potassium bromate. However, black cumin essential was more effective as compared to black cumin fixed oil. The physical examination during the study revealed that experimental diets improved the health of normal rats while balanced functionality of different organs. The findings should be tested in Cohort studies in human subjects for meticulousness.

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