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Protective Effect of Hesperetin on Nicosulfuron-Induced Testicular Oxidative Stress in Male Wistar Rats

[1]OLAYINKA, ET; *[2]KEHINDE, SA; [1]OLAJIDE, AT

[1]Department of Biochemistry, Faculty of Natural Science, Ajayi Crowther University, Oyo, Oyo State, Nigeria.
*[*2]Department of Environmental Health Science, Faculty of Basic Medical Sciences, Ajayi Crowther University, Oyo, Oyo State, Nigeria*

[*]Corresponding Author: E-mail: sa.kehinde@acu.edu.ng; Tel: +2348034499069
Other Authors Email: et.olayinka@acu.edu.ng; at.olajide@acu.edu.ng

ABSTRACT: Nicosulfuron is a post-emergence herbicide used to control weeds while hesperetin found in citrus fruits has been reported to have anti-inflammatory, anti-cancer, and antioxidant properties. This study was designed to investigate the ameliorative properties of Hesperetin on Nicosulfuron-induced reproductive oxidative stress in male Wistar rats. Twenty-four male wistar rats weighing 200 ± 20g were assigned to different groups, each with six animals. Group A serves as the control group and were administered distilled water only. Group B received 25 mg/kg body weight (B.W.) Nicosulfuron. Group C were co-administered with 25 mg/kg B.W. Nicosulfuron and 100 mg/kg Hesperetin while animals in group D received 100 mg/kg B.W. Hesperetin. All treatment lasted for 14 days. An increase in the percentage of sperm with abnormal morphology (23.07%) in the group exposed to Nicosulfuron was observed. Sperm motility, testicular Ascorbic acid, reduced glutathione (GSH) levels were reduced significantly in the Nicosulfuron-treated group by 20.33%, 48.11%, and 41.10% respectively. Also, GST, Catalase, and SOD activities were significantly down-regulated in the Nicosulfuron-treated group. Furthermore, as compared to the control group, the Nicosulfuron-treated group had significantly higher activity of testicular acid phosphatase (ACP), alkaline phosphatase (ALP), MDA, and NO levels. However, co-treatment of Nicosulfuron and Hesperetin significantly ameliorated the Nicosulfuron-induced changes in sperm morphology, motility; testicular ascorbic acid, GSH, NO levels; SOD, CAT, GST, ALP, and ACP activities. The result from this study indicates that Hesperetin, due to its antioxidant properties, protects against testicular oxidative stress induced by Nicosulfuron exposure.

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Nicosulfuron, a member of the sulfonylurea family of herbicides could be regarded as a post-emergence herbicide. It exerts its potential of controlling weeds by inhibiting acetoacetate synthase (ALS) in the plant. Invariably, Inhibiting the ALS enzyme framework will block the synthesis of some amino acids, Valine and isoleucine, basic building blocks of proteins, and other components of plants (Carles *et al.*, 2018). Nicosulfuron is promptly absorbed, albeit only partially (approximately 40%), and is broadly and uniformly distributed. Maximum plasma concentrations were achieved 1-2 hours after oral administration of a low dose, but higher dose levels showed signs of decreased absorption. There were no evidence of accumulating potential. Nicosulfuron is primarily eliminated in the feces (63-73%) and urine (23-28%) and 70 -86 % is mostly excreted unaltered (EFSA, 2007). Nicosulfuron has been reported to have low acute toxicity when administered orally, topically, inhaled, or intraperitoneally. However, there are evidence that alcohol may increase its toxicity (EFSA, 2007). Herbicide exposure can trigger oxidative stress by generating reactive oxygen species (ROS) in an unregulated manner, such as superoxide anion, hydrogen peroxide, hydroxyl radicals, peroxyl radicals, and singlet oxygen. (Yang *et al.*, 2021). It is

[*]Corresponding Author: E-mail: sa.kehinde@acu.edu.ng

suspected that herbicides induce alterations in antioxidants or free oxygen radical scavenging enzyme systems. In addition, it is generally believed that lipid peroxidation is one of the molecular mechanisms involved in herbicide-induced toxicity (Yang *et al.*, 2021). In a healthy body, pro-oxidants and antioxidants remain in balance. Most problems that threaten the health of the reproductive system, especially testicular function, are associated with free radical-induced oxidative stress (Asadi *et al.*, 2017). Life-threatening attacks of free radicals may cause arterial occlusion and serious damage to the reproductive system cells, and consequently defects in spermatogenesis. In other words, increased production of free radicals as well as weakened antioxidant defense systems leads to oxidative stress (Asadi *et al.*, 2017). Hesperetin is a flavanone glycoside found in citrus fruits, it has been reported to have various bioactivities which include antiviral activity, an anticancer activity involving the proliferation of cells. Hesperetin is also a potent antioxidant with a strong inhibitory effect on ROS generation and a free radical scavenger (Ding and Peng, 2015). This study, therefore, evaluates the protective effect of Hesperetin on the toxicity induced by administration of the herbicide, Nicosulfuron, using the testicular functions indices in Wistar rats.

MATERIALS AND METHOD

Chemicals and reagents: Sinochem Ningbo Ltd, 21 Jiangxia Street, Ningbo 315000, China, manufactures Nicosulfuron (Striker®). Sigma Chemical Company produced glutathione (GSH), epinephrine, thiobarbituric acid (TBA), 1-chloro-2, 4-dinitrobenzene (CDNB), 5, 5-dinitrobenzoic acid (DTNB), and para nitro phenyl phosphate (PNPP) (London, UK). The rest of the chemicals and reagents were bought from British Drug House in Poole, London, and were of analytical quality.

Experimental animals: Twenty-four (24) male rats weighing between 200-220g were purchased from the animal house of the College of Medicine, University of Ibadan, Ibadan. The rats were segmented in compartmentalized cages at the animal house, Ajayi Crowther University, Oyo, Nigeria for the period of acclimatization and treatment. The rats were allowed to acclimatize for one week and were allowed free access to feed and water. International principles on the care and use of experimental animals (National Research Council., 2011) were taken into consideration while handling the experimental animals. Animals were assigned into three groups of six rats each: Group A (Control) received distilled water, group B received Nicosulfuron (25mg/kg body weight), Group C received Nicosulfuron (25mg/kg body weight) and Hesperetin (100mg/kg body weight). While Group D received Hesperetin (100mg/kg body weight) only. The route of exposure was oral and treatment lasted for 14 days.

Preparation of Tissue Homogenate: The experimental animals were left for 24hrs after the last administration and sacrificed by cervical dislocation afterwards. Animals were rapidly dissected to excise the testes, rinsed in ice cold 1.15% KCL, blotted and weighed. Testes collected were homogenized in 4 volumes/weight of ice cold 0.1M phosphate buffer pH 7.4. The homogenate was centrifuged at 10,000g for 15 minutes at 4ºC and the supernatant stored at -4˚C and used for subsequent biochemical assays.

Sperm parameters analysis

Testicular and Epididymal Sperm Number, Progressive Sperm Motility Assay, and Volume: Mincing the cauda epididymis and the testis in normal saline and filtering through a nylon mesh were used to collect epididymal and testicular sperm. The spermatozoa were counted using a Neubauer hemacytometer according to Pant and Srivastava's approach, 2003. The motility of epididymal sperm was assessed visually at 400x magnification within 2-4 minutes of being isolated from the cauda. Assessments of mobility were taken from the full field in each sample. The final motility score was calculated using the mean, and the data were reported as percentages. (Diyya *et al.*, 2018).

Morphological and Live-Dead Examination of Spermatozoa: Another slide was used to smear a fraction of the sperm suspension onto a glass slide. The cells were fixed in 95 percent ethanol and stained with 1% Eosin and 5% nigrosine for morphological and viability analyses. At least 100 sperm from each rat were assessed for abnormalities in various spermatozoa regions (Diyya *et al*., 2018).

Assay of biomarkers of testicular injury: The method of measurement of ACP activity was based on that of Wright *et al***.** **(**1972) in which the hydrolysis is determined by spectrophotometric measurement. Alkaline phosphatase activity was determined according to the method described by Wright *et al.* (1972).

Assay of biomarkers of oxidative stress: The ascorbic acid concentration was determined according to the method of Jagota and Dani, (1982).The activity of catalase was determined according to the procedure described by Hadwan and Abed, (2016). Testicular Superoxide dismutase (SOD) activity was determined by the method of Sun and Zigman, (1978). The level

of reduced glutathione (GSH) in the samples was determined by the method described by Jollow *et al*. (1974)**.** Glutathione-S-transferase (GST) activity was determined by the method according to Habig *et al.* (1974). Lipid peroxidation was assayed by measuring the thiobarbituric acid reactive (TBAR) products present in the test sample using the procedure of Vashney and Kale, (1990). The level of Nitric oxide (NO) was determined by the method of Green *et al.* (1982).

Statistical Analysis: The data was presented as a mean standard deviation. Graphpad Prism was used to perform an F-test (ANOVA) on the data acquired (V 8.01). Statistical significance was defined as a P value of less than 0.05.

RESULTS AND DISCUSSION

Table 1 shows the ameliorative effect of Hesperetin on Nicosulfuron-induced changes in sperm morphology of rats. The percentage of rat sperm with abnormal morphology was increased significantly by 23.07% in the Nicosulfuron-treated group as compared to the control group ($p<0.05$). This increase in the percentage of sperm with abnormal morphology was, however, significantly reduced in the Nicosulfuron and Hesperetin co-treated group when compared to the Nicosulfuron group ($P<0.05$). The ameliorative effect of Hesperetin on Nicosulfuron-induced changes in sperm parameters of rats were presented in table 2. Percentage of sperm motility was significantly decreased by 20.33% respectively in Nicosulfuron-treated group when compared to the control group ($p<0.05$). However, the decrease in sperm motility was significantly ameliorated when Hesperetin was co-treated with nicosulfuron.

Ameliorative effect of Hesperetin on Nicosulfuron-induced changes in testicular ascorbic acid and reduced glutathione (GSH) levels in rats is shown in Table 3. Ascorbic acid and GSH levels were significantly decreased by 48.11% and 41.10% respectively in Nicosulfuron-treated group relative to the control group ($p<0.05$). However, the ascorbic acid and GSH levels were significantly increased in the Nicosulfuron and Hesperetin treated group ($p<0.05$) compared with the Nicosulfuron group. Table 4 present the ameliorative effect of Hesperetin on Nicosulfuron-induced changes in testicular superoxide dismutase (SOD), catalase and glutathione s-transferase (GST) activities. SOD, CAT, and GST activities were significantly decreased in the Nicosulfuron-treated group by 47.89%, 43.93% and 50.16% respectively as compared to the control group ($P<0.05$). However, co-administration of Nicosulfuron and Hesperetin significantly attenuated the activities of SOD, catalase and GST relative to the Nicosulfuron-treated group.

Table 1: Ameliorative Effect of Hesperetin on Nicosulfuron-Induced Changes in Sperm Morphology of Rats

Treatment	% of sperm with normal morphology	% of sperm with abnormal morphology
Control	88.93±0.45	11.07±0.45
NICO	85.61±0.46 (3.67%)*	14.39±0.46 (23.07%)*
NICO + HES	87.42±0.14*	12.58±0.15*****
HES	88.12±0.22*** [a]**	11.88±0.22*** [a]**

*NICO= Nicosulfuron (25mg/kg body weight); HES= Hesperetin (100mg/kg body weight). The results are expressed as Mean ± SD for six rats in each group. * Significantly different from the control group (P<0.05) [a] significantly different from the nicosulfuron group (P<0.05). Values in parenthesis represent percentage (%) decrease or increase.*

Table 2: Ameliorative Effect of Hesperetin on Nicosulfuron-Induced Changes in Sperm Parameters of Rats

Treatment	Motility (%)	Live/dead (%)	Count (x10^6/ml)	Volume (cc)
Control	91±2.24	97.4±1.34	414±2.37	5.16±0.054
NICO	72.5±5.01 (20.33%)*	96.5±1.73 (0.93%)	401.25±2.5 (3.08%)	5.15±0.05 (0.19%)
NICO + HES	82±2.74*	96.5±1.64	408.33±2.08	5.2±0
HES	84±2.24*[,a]	96.5±1.64	410.83±2.04	5.18±0.044

*NICO = Nicosulfuron (25mg/kg body weight); HES = Hesperetin (100mg/kg body weight). The results are expressed as Mean ± SD for six rats in each group * significantly different from the control group (P<0.05) [a] significantly different from nicosulfuron group (P<0.05). Values in parenthesis represent percentage (%) decrease.*

Table 3: Ameliorative Effect of Hesperetin on Nicosulfuron-Induced Changes in Testicular Ascorbic Acid and Reduced Glutathione (GSH) Levels in Rats

Treatment	Ascorbic acid (µg/mL)	GSH (µg/g testis)
Control	1.85±0.02	7.30±0.1
NICO	0.96±0.02 (48.11%)*	4.3±0.08 (41.10%)*
HES	1.82±0.01 *	7.1±0.07 *
NICO + HES	1.05±0.02 *[,a]	6.64±0.11 *[,a]

*NICO = Nicosulfuron (25mg/kg body weight); HES = Hesperetin (100mg/kg body weight). The results are expressed as Mean ± SD for six rats in each group * significantly different from the control group (P<0.05) [a] significantly different from nicosulfuron group (P<0.05). Values in parenthesis represent percentage (%) decrease.*

Table 4: Ameliorative Effect of Hesperetin on Nicosulfuron-Induced Changes in Testicular Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione S-Transferase (GST) Activities in Rats

Treatment	Unit SOD	Catalase (µmoL H_2O_2 consumed/min)	GST (nmoL/min/mg protein)
Control	1.42±0.01	28±0.71	77.75±0.5
NICO	0.74±0.02 (47.89%) *	15.7±0.26 (43.93%)*	38.75±0.96 (50.16%)*
HES	1.39±0.01	27±0.15 *	74±0.82
NICO + HES	1.32±0.02 *,a	23.92±0.11 *,a	66±0.82 *,a

*NICO = Nicosulfuron (25mg/kg body weight); HES = Hesperetin (100mg/kg body weight). The results are expressed as Mean ± SD for six rats in each group * significantly different from the control group (P<0.05) [a] significantly different from nicosulfuron group (P<0.05). Values in parenthesis represent percentage (%) decrease.*

Hesperedin ameliorates Nicosulfuron-induced alterations in alkaline phosphatase (ALP) and acid phosphatase activity (ACP): The ameliorative effect of Hesperetin on nicosulfuron-induced alterations in activities of alkaline phosphatase (ALP) and acid phosphatase (ACP) in the testes of rats was shown in figure 1. There were significant decrease in ALP and ACP activities in Nicosulfuron-treated group relative to the control (P<0.05). Combined treatment of Nicosulfuron and Hesperetin was seen to significantly protect the decrease in testicular ALP and ACP activities when compared with Nicosulfuron-treated group.

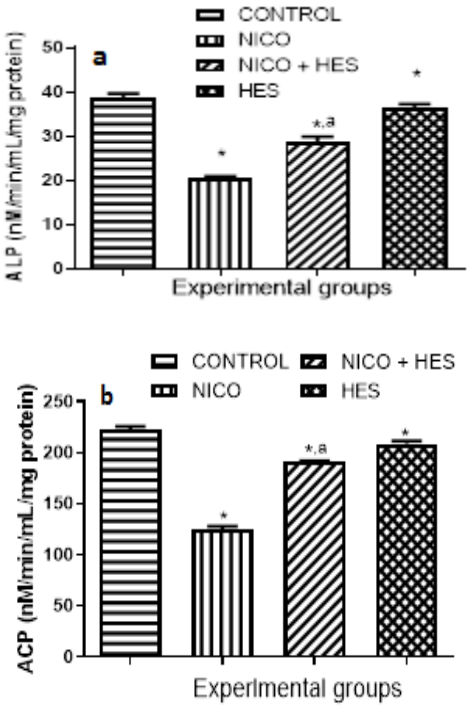

Fig 1: ameliorative effect of Hesperetin on nicosulfuron-induced alterations in activities of (a) alkaline phosphatase (ALP) and (b) acid phosphatase (ACP) in the testes of rats.
*NICO = Nicosulfuron (25mg/kg body weight), HES = Hesperetin (100mg/kg body weight). Each bar represents the mean ± SD (n=6). * - significantly different compared with control (P < 0.05). a- significantly different compared with nicosulfuron (P<0.05).*

Hesperetin ameliorates nicosulfuron-induced alterations in malondialdehyde and nitric oxide level: Figure 2 shows the ameliorative effect of hesperedin on nicosulfuron-induced alterations in levels of nitric oxide (NO) and malondialdehyde (MDA) in the testes of rats. There were significant increase (p<0.05) in the level of MDA and NO in the testes of nicosulfuron-treated rats when compared with control. However, coadministration of Hesperetin and nicosulfuron significantly ameliorated the alteration relative to nicosulfuron-treated group.

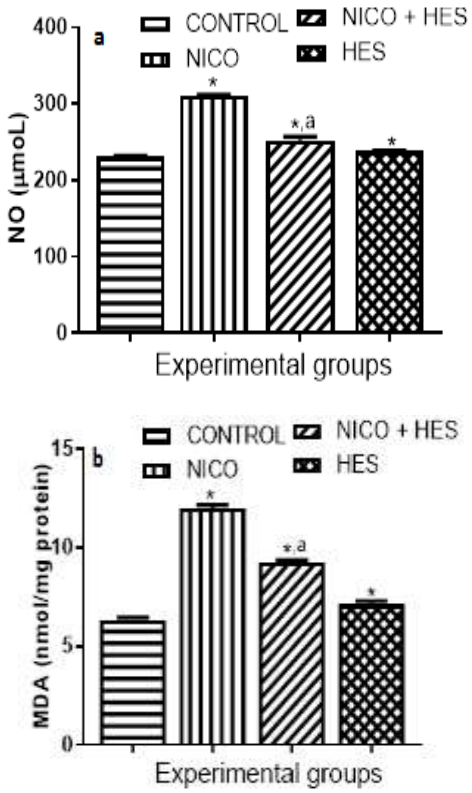

Fig 4: ameliorative effect of Hesperetin on nicosulfuron-induced alterations in levels of (a) nitric oxide NO) and (b) malondialdehyde (MDA) in the testes of rats.
*NICO = Nicosulfuron (25mg/kg body weight); HES = Hesperetin (100mg/kg body weight); Each bar represents the mean ± SD (n=6). * - significantly different compared with control (P < 0.05). a- significantly different compared with nicosulfuron (P<0.05).*

This study investigated the ameliorative effect of Hesperetin on nicosulfuron-induced testicular oxidative stress in rats. Nicosulfuron is an herbicide that acts by inhibiting the plant enzyme acetolacetate synthase and may result in oxidative stress, upon

exposure, to Humans and other animals (Carles *et al.*, 2018). Hesperetin is a potent antioxidant with strong inhibitory effect on reactive oxygen species generation and is a free radical scavenger (Ding and Peng, 2015). From this study, it was observed that Nicosulfuron caused impairment of the male reproductive system as seen in the increased percentage of sperm with abnormal morphology and reduced percentage motility in the Nicosulfuron-treated group. This induced alteration was ameliorated by Hesperetin. Therefore, Hesperetin may protect against disruptions of male reproductive end points caused by Nicosulfuron. Alkaline phosphatase and acid phosphatase are markers of testicular toxicity. The activities of these testicular markers are considered functional indicators of spermatogenesis. In the spermatogenic cells, the specific activity of ACP increases as the germ cells differentiate from spermatogonia into spermatocytes and spermatids (Olayinka and Ore, 2014). A decrease in the activities of both testicular ALP and ACP in the Nicosulfuron-treated group observed in this study, accounts for the increase in the percentage of sperm with abnormal morphology. However, Hesperetin significantly attenuated the induced reduction of ALP and ACP activities, thereby promoting spermatogenesis. Herbicide exposure can cause oxidative stress by releasing reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radicals, peroxyl radicals, and singlet oxygen in an uncontrolled manner (Yang *et al*., 2021). In biological systems, ROS can be both damaging and beneficial depending on the environment and concentration (Pizzino *et al*., 2017). However, at high concentrations, ROS can cause damage to cell components such as membrane lipids, proteins, and nucleic acids, which is known as "oxidative stress" (Pizzino *et al*., 2017). As a result, oxidative stress in the male reproductive organs may damage cells, resulting in a reduction in sperm fertility by changing sperm count, motility, morphology, and other factors.

Antioxidants, enzymic and non-enzymic ones are capable of deactivating these free radicals when there is a balance between oxidants and antioxidants. Exposure to nicosulfuron depleted the levels of GSH, AA and activities of CAT, SOD and GST relative to the control group establishing the oxidative stress inducing capacity of the herbicides which may disrupt both spermatogenesis and the production of testosterone. However, co-administration of Hesperetin and nicosulfuron ameliorated these observed alterations in the antioxidant system.

Superoxide dismutase (SOD), catalase (CAT) and glutathione-s-transferase (GST) are enzymatic antioxidants involved in scavenging free radicals or reactive oxygen species. SOD catalyzes the reaction that converts superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2), preventing the O_2^- from producing highly harmful hydroxyl radicals. The H_2O_2 produced in this way is a powerful membrane permeant oxidant in and of itself, and it must be quickly removed from the cell to avoid oxidative damage to lipids, proteins, and DNA. (Wang *et al.*, 2018; Shin *et al.*, 2018). Glutathione-S-Transferase (GST) is an enzyme involved in the detoxification of ingested xenobiotics and catalyzes the reduction of peroxide-containing compounds in the cell. This peroxidase activity exhibited by GST is however dependent on availability of GSH (Ajayi *et al.*, 2022). On the other hand, ascorbic acid (AA) scavenges free radicals and function in the regeneration of a membrane bound antioxidant, vitamin E (Njus *et al.*, 2020). The elevation of malondialdehyde (MDA) level is an indication of lipid peroxidation which can be caused by ROS attack. The measure of lipid peroxidation is a convenient method to monitor oxidative stress, as membrane lipids succumb easily to deleterious actions of reactive oxygen species (Tsikas, 2017). The elevation of MDA level may be due to the effect of H_2O_2, $^{\bullet}OH$ and $ONOO^-$ radicals which interact with polyunsaturated fatty acids in the phospholipids of cell membrane, inducing lipid peroxidation in testis tissues (Žaja *et al.*, 2016). In this study, it was observed that MDA level was significantly increased in the Nicosulfuron-treated group, thus, implying that Nicosulfuron induced in lipid peroxidation and therefore, oxidative stress in testis. This increase in MDA level was seen to be significantly attenuated with treatment with Hesperetin, indicating that Hesperetin decreased lipid peroxidation and oxidative stress thus leading to reduction of testicular injuries. Also observed in the Nicosulfuron-treated group was the increase in nitric oxide (NO) level. NO has been associated with germ cell necrosis and destruction of testis (Zhang *et al.*, 2013). The oxidative stress induced by Nicosulfuron was seen to be ameliorated in rats administered with Hesperetin and nicosulfuron.

Conclusion: From this study, it has been established that nicosulfuron induces testicular oxidative stress and may subsequently lead to impairment of the key functions of the male reproductive system. Hesperetin was found to ameliorate these deleterious effects of Nicosulfuron exposure and thus, consumption of plant sources rich in Hesperetin may help reduce testicular oxidative toxicity caused by herbicide exposure.

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