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PHYTOCHEMICAL ANALYSIS AND *IN VITRO* EVALUATION OF ANTIFERTILITY ACTIVITY OF *CHENOPODIUM AMBROSIOIDES* LEAF EXTRACT

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ABSTRACT

Chenopodium ambrosioides is herbaceous plant species that possess natural substances having anti-androgenic properties and can be used as source of contraception. Current study was designed to explore the phytochemical analysis and assessment of antifertility activity of *C. ambrosioides* leaf extract in adult male rats. Methanolic extract was evaluated for total phenolic contents (TPC), total flavonoid contents (TFC), antioxidant potential and GC-MS analysis was carried out to scrutinize bioactive phyto-constituents in the extract. For assessment of antifertility activity, in vitro experimental approach was used to see direct effect of different concentrations of plant extract on sperm DNA integrity, antioxidant status and testosterone secretion in testis. Results showed presence of adequate amount of total flavonoid and phenolic contents along with antioxidant potential. GC-MS analysis of methanolic leaf extract showed presence of thirteen bioactive compounds in *C. ambroisoides.* In the in vitro experiment, highest dose regimen (1000 µg/mL) treatment indicated considerably increased oxidative stress and reduced antioxidant activity. This increased concentration of ROS and lipid peroxidation result in DNA damage in the rat sperm. Likewise, decrease in testicular testosterone concentration was noted after two hours incubation with all the selected doses of *C. ambrosioides* leaf extract. The findings of current study recommend that *C. ambrosioides* have the potential to disturb male fertility by inducing oxidative stress and hormonal imbalance in rat testis and disrupting sperm DNA integrity when exposed to higher concentrations of extracts.

INTRODUCTION

World population has been enormously increasing, affecting environment, health and economic growth in under developed as well as developing countries (Thakur *et al*., 2010). These circumstances would increase the need for effective measures. Subsequently, several attempts have been made to control the birth rate by various means, thus, causing several deleterious side effects such as hormonal imbalance, hypertension, and weight gain (McNamara, 2015). Hence, there is a necessity to replace these substances by safe and effective alternative like plant-based contraceptive agents.

The use of plants and herbs for fertility regulation has been prevalent worldwide for many centuries. They possess natural substances having androgenic and anti-androgenic properties and can be used as source of contraception with fewer side effects. (Priya *et al*., 2012). Development of safe, effective and reversible male contraceptive with no effect on sexual activities or libido is challenging. Antifertility activity of a variety of plants having terpenes, flavonoids, quinines and tannins has been revealed previously (Ain *et al*., 2018; David *et al*., 2019; Joshi *et al*., 2011). Plants of Chenopodiaceae family are known to be enriched with, flavonoids, phenols, saponins and alkaloids (Ibrahim *et al*. 2007; Kokanova-Nedialkova *et al*. 2009) that might cause spermatogenic arrest leading to fertility suppression.

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Chenopodium ambrosioides, commonly known as 'Skhabotay (Kamasal Bhang)', is an aromatic perennial shrub of family Chenopodiaceae that is extensively cultivated worldwide. Although little is known about toxicological profiles of *C. ambrosioides*, but it has long been used in traditional medicines as a dietary condiment. In a previous study, a fairly heavy consumption of essential oil of *C. ambrosioides* has been associated with intoxication in rats as well as in humans (Ruffa *et al*., 2002). These toxic effects are more likely due to the presence of terpenoids that despite of diverse pharmacological properties, also have toxic aspects (Kiuchi *et al*., 2002; Liu, 2004). Moreover, *C. ambrosioides* is also toxic for several insects and potentially used as a botanical insecticide (Tavares and Vendramim, 2005). In another study, aqueous seed extract of closely related species, *C. Album* immobilized spermatozoa and disrupted sperm plasma membrane in rats and rabbits when exposed to varied concentrations of extract by using *in vitro* approach (Kumar *et al*., 2007). It was previously shown that aqueous extract of *C. ambrosioides* negatively influence the reproduction in Drosophila melanogaster (Wohlenberg and Lopes-da-Silva, 2009). Previously, we have evaluated in vivo antifertility potential of *C. ambrosioides* using adult male rats (Ain *et al*., 2018). So on the basis of previous study,

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it can be hypothesized that *C. ambosioides* extract can suppress fertility in *in vitro* conditions.

Given the lack of literature and knowledge along with its extensive and widespread traditional use, the purpose of current study is phytochemical analysis and antifertility assessment of *C. ambrosioides* leaf extract on testicular antioxidant status and testosterone production as well as DNA integrity in rat spermatozoa by using in vitro experimental approach.

MATERIALS AND METHODS

Plant collection and extract preparation

Fresh leaves of *C. ambrosioides*, were obtained from agricultural and cultivated fields of Abbottabad. The plant was selected on the basis of its extensive medicinal use in respective site of collection and also in different areas of Pakistan.

Leaves of selected plant were separated from stem, air-dried, ground in waring blender and then sieved. Leaf powder was soaked in 99.9% methanol (leaves to solvent ratio 1:10) for seven days. The extract was filtered using Whattman filter paper and concentrated on a rotary evaporator (Model: Hei-VAP Heidolph, Germany) according to previously reported method described by Gulfraz *et al*., 2007 (Gulfraz *et al*., 2007). The filtrate mass was dried at room temperature and stored at 4ºC.

Phytochemical analysis

Total flavonoid and Phenolic contents estimation

For the determination of total flavonoids content, aluminum trichloride ($AlCl3$) colorimetric method was used as described earlier with slight modifications Quettire-Deleu *et al*. (Quettier-Deleu *et al*., 2000). While total phenolic contents were assessed by using Folin-Ciocalteu method as described earlier by Jagadish *et al*. (Jagadish *et al*., 2009) with slight modifications.

Antioxidant potential estimation DPPH assay

Free radical scavenging activity was assessed through DPPH assay using method explained by Tai *et al*. with slight modifications (Tai *et al.,* 2011) Antioxidant activity of selected plants extracts was evaluated by their capability to reduce the stable 2, 2-diphenyl 1-picrylhydrazyl (DPPH). IC_{50} values were calculated and expressed as µg AAE/mg of extract. Percent radical scavenging activity was determined by following formula:

$$\%RSA= [1 − (OD\ of\ Extract)/(OD\ of\ Control)] \times 100$$

Total antioxidant capacity determination

To determine total antioxidant capacity of the plants extracts, phosphomolybdenum method was used. In this method, green colored phosphate/Mo (V) complex is made when Mo (VI) is reduced to Mo (V) (Prieto *et al*., 1999).

Gas chromatography-mass spectrometry analysis (GCMS)

The GCMS analysis was carried out by using GCMS-QP5050 Shimadzu, Japan. The capillary column used was DB-5/RTX-MS (30 m length and 0.25 mm diameter consisting of 95% dimethyl polysiloxane). Helium was used as carrier gas at flow rate of 1 ml/min with linear velocity of 37.2 cm/sec and 1 µL injection volume. For sample analysis, column temperature was maintained at 90°C for 1 min after injection.

Injector temperature was elevated to 200°C with 10°C increase in every minute. Final temperature was elevated to 250°C with 10°C increase in temperature/min for 15 min. Temperature of detector was maintained at 300°C while that of injector at 250°C. An election ionization system was used for detection with ionization energy of 70eV. Pressure was maintained at 60.0 kPa and sample was run for 60 min. A scan rate of 0.50 s (cycle time: 0.2 s) was applied, which covers a mass ranging 35 to 600 amu (Gomathi *et al*., 2017).

Animals

Twenty four adult male Sprague Dawley rats (70 - 90 days old) were obtained from the primate facility of Animal Sciences Department, Quaid-i-Azam University, Islamabad. Animals were randomly placed in stainless steel cages and kept in well ventilated room. Temperature of 26±1 ºC and 10 /14 h of dark and light cycles were set for acclimatization. Animals were provided with standard laboratory made feed and water was available in plastic bottles. All the experimentation and protocols were ratified by ethical committee of Animal Sciences department, QAU.

In vitro experiment

Twenty four adult male Sprague Dawley rats were used for this study. Experimental design was made according to Moundipa *et al*. (2006) with some modifications (Moundipa *et al*., 2006). To best of my knowledge, there is no reported *in vitro* study using methanolic extracts of this plant, so the range of doses were selected according to OECD protocols and previous *in vitro* studies using plant extracts as recommended by Srivastav *et al*. (2010) (Srivastav *et al*., 2010). Five different doses (0, 1, 10, 100 and 1000 µg/mL) were selected for this study. Stock solution of plant extracts were prepared in methanol and was further mixed with cell culture media. The concentration of methanol was retained less than 0.5% in prepared media.

In vitro culturing of testis was executed as described earlier by Moundipa *et al*. (2006), Erasmus *et al*. (2012) and Srivastav *et al*. (2010) with little modifications (Moundipa *et al*., 2006; Srivastav *et al*., 2010; Erasmus *et al*., 2012). Rats were decapitated and both testes were obtained and washed with cold physiological saline. After decapsulation, each testis was sliced into six fragments having almost equal. In autoclaved culture tubes, testicular slices were added along with 2 ml of culture media (DMEM/Ham F12), sodium bicarbonate (1.2 g/L) and antibiotics i.e. penicillin (50 IU/mL) and streptomycin (50 µg/mL). Subsequently, selected doses

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of plant extract (0, 1, 10, 100, 1000 µg/mL of each plant extract) were added in each culture tube. Tubes were incubated for 2 hours at 37 °C in CO_2 incubator (with 5% CO_2 and 95% air (v/v)). After incubation, tissues were removed from media, washed and homogenized in 1 mL of PBS (pH= 7.4), centrifugation was done at 30,000 rpm for 30 min. Supernatant was separated and stored at -80 °C until further analysis of biochemical and hormonal assays.

For the *in vitro* culturing of sperm, epididymis was separated immediately after dissection and washed with saline. With the help of sharp scissor, cauda part was crushed and homogenized in 3 mL of a buffer (150 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L Tris base, 10% glycerol with pH=7.4). The homogenates were centrifuged for 10 minutes at 4 ºC and supernatant was removed. Ham's F12 media along with bovine serum albumen was added in sperm pellets. Culture tubes, containing 2 ml of sperm suspension and 2 ml of media with 0, 1, 10, 100 & 1000 µg/mL of plant extract, were incubated in CO_2 incubator for 2 hours. Temperature was maintained at 37 ºC and 5% CO_2 was set. After 2 hours of incubation, centrifugation was done for 15 minutes at 1000 rpm and supernatant was removed leaving behind sperm pallet. The sperm pellets were diluted with 1 mL of phosphate buffer saline and used for comet assay.

Antioxidant enzyme status

Following centrifugation of testicular homogenate, supernatant was used for determination of antioxidant enzymes status.

Superoxidase dismutase assay (SOD)

Superoxidase dismutase activity was evaluated by previously reported procedure by Kakkar *et al.* (1984) (Kakkar *et al*., 1984). A reaction mixture was prepared by adding phenazinemethosulphate (18 uM, 0.1 ml), sodium pyrophosphate buffer (0.052 mM, 1.2 ml with pH 7.0) and 0.3ml of homogenate. Reaction was started by addition of NADH (0.2 ml, 780 uM), and stopped after 60 sec by the addition of 1 ml of glacial acetic acid. Change in absorbance of reaction mixture was related as to change in colour intensity of chromogen formed. Absorbance was noted at 560 nm wavelength by using by spectrophotometer. Value was given in units/mg protein.

Peroxidase assay (POD)

Peroxidase (POD) activity was assessed by previously recommended procedures of Chance and Maehly, (1955) in their respective studies (Chance and Maehly, 1955). Reaction mixture was prepared in cuvette containing 1000µl of homogenate, PBS (2.5 ml, 50 mM, pH= 5.0), guaiacol (0.1 ml, 20 mM) and H_2O_2 (0.3 ml, 40 mM). After one minute, absorbance was recorded at 470 nm. The value of POD was expressed in mU/mg protein.

Catalase assay (CAT)

Catalase activity was assessed by already reported method of Chance and Maehly (1955) with some amendments (Chance and Maehly, 1955). In the cuvette, reaction mixture was made by adding PBS (2.5 ml, 50mM, pH 5.0), supernatant (0.1 ml) and H_2O_2 (0.4 ml, 5.9 mM). After one minute, absorbance of reaction mixture was recorded at 240 nm by using spectrophotometer. Absorbance change of 0.01 unit/minute was called one unit of CAT activity.

Estimation of lipid peroxidation assay (TBARS)

Lipid peroxidation in testicular homogenate was estimated by using procedure given by Iqbal *et al.* (1996) (Iqbal *et al*., 1996). In the test tube, PBS (0.58 ml, 0.1M pH 7.4) was dispensed along with ascorbic acid (0.2 ml, 100mM) and ferric chloride (0.02 ml, 100mM). After that 0.2 ml homogenate was incorporated in the mixture and incubated at 37 ºC for 1 hour. Following incubation, reaction was stopped by addition of 1.0 ml of trichloroacetic acid (10%). After that 1.0 ml of thiobarbituric acid (0.67%) was dispensed in all the tubes and again incubated in boiling water for about 30 min before moving to crushed ice. Samples were centrifuged at 2500 × g for 10 min. Absorbance of mixture was recorded at the wavelength of 535 nm.

Estimation of Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) concentration was determined in testicular tissue by using procedure reported previously by Hayashi *et al.* (2007). Standards of H_2O_2 were prepared by dilutions and 5 µL of standard and homogenate were dispensed in 96 well plate along with 140 µL of sodium acetate buffer (0.1 M, pH= 4.8) and incubated at room temperature for 5 min. A solution of N, N-diethyl-para-phenylenediamine (DEPPD) and ferrous sulphate (1:25) was prepared and incubated in dark for 20 min. 100 µL of this solution was dispensed in well and mixed thoroughly. Absorbance was recorded for 90 s with 15 s interval by using microplate reader at 505 nm. One unit of ROS was equal to hydrogen peroxide concentration in the sample.

Estimation of DNA damage

DNA damage of each spermatozoa was determined by using a neutral single cell electrophoresis (SCGE / comet assay) using procedure described by Boe-hensen *et al.* (2007) (Boe-Hansen *et al*., 2006). Frosted microscopic slides were coated with 100µL of 1% regular melting point agarose (RMPA), covered with coverslip and left at 4ºC until the solidification of agarose. After that, a mixture of 20 µl of sperm homogenate and 65µl of 1% low melting point agarose (LMPA) was prepared and spread over the first one. The slides were covered and let the agarose to solidify. Slides were then incubated at room temperature with freshly prepared lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mMTris base, 1% (w/v) Triton X-100, pH =10.3). After 24 hours, slides were removed and washed with saline so that any salt or chemical traces can be washed away. In the electrophoresis tank, electrophoresis buffer (54 g/L Tris

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base, 0.5 M EDTA, 27.5 g/L boric acid, pH=8.0) was poured and slides were orderly arranged. Electrophoresis was conducted for 20 min at 25V (0.714 V/ cm). After the completion of procedure, slides were removed from the tank, air dried and covered in dark to avoid the light.

Before analysing, the slides were rehydrated with distilled water and stained with acridine orange. Slides were examined under fluorescent microscope (40 X, AFX - 1 Optiphot, Nikon, Tokyo, Japan) and digital photographs were taken for further analyses by using comet assay software Casplab, V. 1.2.3b2. The numbers of comets/100 spermatozoa were counted and head length (HL, µm), Tail length (TL, µm), head DNA (%), Tail DNA (%) and tail moment (µm) were noted.

Hormonal Analysis

Testosterone level was determined quantitatively in testicular homogenate by Enzyme Linked Immuno Sorbant Assay (ELISA) kits (Biocheck Inc, USA) by using the manufacturer's instructions.

Statistical analysis

Data was analyzed by one way analysis of variance (ANOVA) followed by Dunnet's multiple comparison test. Various dose groups were compared with control by using Graph Pad Prism 5 software. Level of significance was set at $p<0.05$.

RESULTS

Determination of total flavonoid and total phenolic contents

The methanolic leaf extracts of *C. ambrosioides* possess considerable range of flavonoid and phenolic contents as shown in Table 1.

Antioxidant potential Estimation

Free radical scavenging activity of the methanolic leaf extract of *C. ambrosioides* was estimated by using DPPH assay. IC_{50} value was also calculated for evaluation of results. It was observed that that methanolic leaf extract possesses free radical scavenging activity with inhibitory concentration (IC_{50} value) of 56 µg/mL as shown in table 1.

Total antioxidant activity of methanolic leaf extract of *C. ambrosioides* was also investigated and results are given in Table 1.

Gas chromatography-Mass spectrometry (GC-MS) analysis

The details of different compounds identified by GC-MS analysis of methanolic leaf extract of *C. ambrosioides* are given in Table 2 and GC-MS chromatogram is given in figure 2.

These compounds are of different nature including fatty acid methyl ester, amino acids and phytol. The most abundant compounds found in present study were Arginine with percent area of 9.19% followed by Benzeneacetic acid,2,5-dihydroxy (8.02%), 9-Eicosene (5.91%), 4-Nonenoic acid (4.54%), 1-Heptadecene (3.29%), 1-Tridecene (3.24%) and Tetradecanoic acid (2.48%) along with minor constituents.

Table: 1. Total flavonoid contents (TFC), total phenolic contents (TPC), total antioxidant capacity (TAC) and DPPH free radical scavenging activity of methanolic leaf extract of *C. ambrosioides* with IC50 value.

Parameters	Plant Extract
TPC (µg GAE/mg)	227.12±1.76
TFC (µg QE/mg)	146.42±0.95
TAC (µg AAE/mg)	291.37±1.13
DPPH free radical scavenging activity (%)	
400 µg/mL	88.90
300 µg/mL	85.44
200 µg/mL	77.85
100 µg/mL	60.87
IC_{50} value	56 ug/ml

Effect of *C. ambrosioides* on testicular antioxidant status and testosterone secretion

Biochemical and hormonal profile of testicular tissue of rat was determined after incubation for two hours with different concentrations of methanolic leaf extract of *C. ambrosioides* (Table 3). A dose dependent reduction in catalase activity was seen in high dose treated group. The reduction was significant in 10 µg/mL and 1000 µg/mL dose treatment groups as compared to control ($p<0.01$ and $p<0.01$ respectively). Similarly, SOD and POD efficacy of methanolic leaf extract of *C. ambrosioides,* on the reproductive system of male rats by using in vitro experimental approach.

Results of phytochemical analysis revealed that considerable amount of total flavonoid contents in term of quercetin equivalent and total phenolic contents in term of gallic acid were found in methanolic extract. These results were similar to previously reported studies in which crude extract of H. nepalensis and its fractions showed high amount of phenols and flavonoid contents (Jafri *et al*., 2017). Flavonoids and phenols are strongly associated with antioxidant activity of biological system and play important role to stabilize lipid oxidation (Rahman *et al*., 2017).Determination of free radical DPPH scavenging activity is cheap method for the assessment of antioxidant potential. This process is relied on conversion of purple colored DPPH into yellow colored 2,2-diphenyl-1-picryl-hydrazyl by receiving electron from antioxidants.

Another parameter for determination of free radical scavenging potential is IC_{50} value. Low IC_{50} specifies substantial free radical scavenging activities (Rahman *et al*., 2017). In our study, *C. ambrosioides* exhibited total

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antioxidant capacity with 291 µg/mg with high inhibitory concentration (IC_{50} value) of 56 µg/mL. These results showed that although *C. ambrosioides* extract possess antioxidants activity but free radical scavenging potential is low as comprehended by high IC_{50} value.

In present study, phytochemical components of methanolic leaf extract of *C. ambosioides* were analyzed by GC-MS. Some identified compounds present in methanolic leaf extract of *C. ambrosioides* include many saturated fatty acids, lipids, amino acids and phytol. Some of the compounds are known for anticancer, antimicrobial and anti-inflammatory activities (Pandey and Gupta, 2014). Lipid and fatty acids are involved in the generation of systemic oxidative stress, a well-recognized deleterious factor for the sperm quality and associated with infertility (Collodel *et al*., 2020) supporting hypothesis of present study.

It is well known that oxidative stress induced by ROS and RNS disturb cellular homeostasis, dysregulates signaling network, damage proteins, nucleic acids and lipids leading to genomic instability (Weyemi and Dupuy, 2012). Excessive accumulation of ROS/RNS, lead to various diseases including cardiovascular diseases, neurodegeneration, hypertension, diabetes, atherosclerosis and rheumatoid arthritis (Phaniendra *et al*., 2015). Testes are known to be more profound to oxidative stress because of presence of reactive oxygen species and unsaturated fatty acids responsible for lipid peroxidation (Ain *et al*., 2018). The findings of current study showed that exposure of testis to different concentrations of *C. ambrosioides extract* caused reduction in testicular antioxidant levels (SOD, POD, CAT) and increase in ROS and TBARS. However, this oxidative stress was more prominent in tissues incubated with high dose regimens. This plant extract induced oxidative damage can impair spermatogenesis and steroidogenesis as reported earlier in the *in vivo* exposure of *C. ambrosiodes* extract to adult male rats (Ain *et al.,* 2018).

Results of comet assay in current study indicated that methanolic extract of *C.ambrosioides* affect sperm DNA after exposure with higher doses (100 and 1000 µg/mL). Previously, various *in vitro* and *in vivo* studies have shown association of oxidative stress with testicular dysfunction and sperm DNA damage (Kumar *et al*., 2012). Similar findings were reported earlier, in which administration of fenugreek seed to mice reduced fertility in dose dependent manner by affecting sperm parameters and producing abnormal sperms with DNA damage (Al-Yahya, 2013). Kumar *et al*. (2002) proposed association between DNA damage and oxidative stress leading to infertility in male mice (Kumar *et al*., 2002).

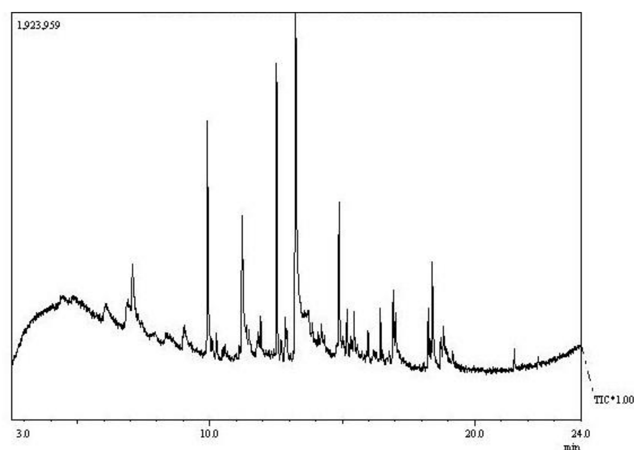

Figure 1: GC/MS chromatogram of methanolic leaf extract of *C. ambrosioides*

In present study, incubation of testicular tissues with methanolic leaf extract of *C. ambrosioides* reduced testicular testosterone concentrations. Normal circulating levels of testosterone are necessary for spermatogenesis and fertility regulation. Increased oxidative stress in testicular tissue might disrupt steroidogenesis by Leydig cells reducing hormonal levels (Hales *et al*., 2005).

CONCLUSION

The findings of current study revealed that methanolic leaf extract of *C. ambosioides* possess considerable amount of flavonoids and phenolic contents with antioxidant potential. It possesses a variety of components, as identified by GC-MS analysis, which could be used as novel scaffolds in contraceptive drug discovery research. This study provides basis and direction for the further pharmacological investigations of studied plant. Further, results of *in vitro* experiment revealed that methanolic leaf extract of *C. ambosioides* has the potential to induce oxidative stress in testicular tissue associated with reduction in testicular testosterone and sperm DNA damage. It is suggested that *C. ambrosioides* plant extracts might have stage specific genotoxic effect on germ cells and can be used to suppress fertility by producing oxidative stress and damaging sperm DNA.

Declaration of interest

The authors report no declarations of interest.

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Table 2: Compounds identified from methanolic leaf extract of *C. ambrosioides* through GC-MS analysis.

Retention Time (min)	Name of Compound	Molecular formula	Molecular weight	% Area	% Height	Cas #
7.083	Arginine	C6H14N4O2	174	9.19	5.45	74-79-3
9.908	1-Tridecene	C13H26	182	3.24	7.31	2437-56-1
11.217	4-Nonenoic acid	C10H18O2	170	4.54	8.09	20731-19-5
12.517	1-Heptadecene	C17H34	238	3.29	12.62	6765-39-5
13.233	Benzeneacetic acid,2,5-dihydroxy	C8H8O4	168	8.02	17.81	451-13-8
14.875	9-Eicosene	C20H40	280	5.91	7.12	74685-29-23
15.183	Pterin-6-carboxylic acid	C7H5N5O3	207	-	-	948-60-7
15.450	Hexadecanal	C16H32O	240	-	-	629-80-1
15.975	Pterin-6-carboxylic acid	C7H5N5O3	207	0.29	0.21	948-60-7
16.433	Hexadecanoic acid, methyl ester	C17H34O2	270	0.68	2.66	112-39-0
16.925	Tetradecanoic acid	C14H28O2	228	2.48	3.67	544-63-8
18.250	11-Octadecenoic acid, methyl ester	C19H36O2	296	2.19	2.14	56554-45-1
18.400	Phytol	C20H40O	296	1.95	1.72	150-86-7

Table: 3. Mean ± SEM of on specific activity of testicular antioxidant enzymes, ROS, TBARS and plasma testosterone concentrations of control and *in vitro* extract treated groups.

Parameters	Extract Treatment (µg/mL)				
	Control	1	10	100	1000
CAT (U/mg protein)	4.46±0.60	4.33±0.53	2.19±0.70*	2.76±0.55	1.25±0.32**
SOD (U/mg protein)	20.37±2.38	15.12±2.32	19.68±2.38	16.71±3.28	14.63±1.98*
POD (nmole)	11.54±1.08	12.31±1.27	10.55±1.54	12.56±1.28	6.84±1.48*
TBARS (nM/mg tissue)	19.32±1.81	18.68±1.72	20.67±3.07	22.01±3.04	25.37±2.92
ROS (U/g tissue)	13.51±1.15	14.09±1.79	22.05±3.41	29.39±4.87**	34.85±3.41***
Testosterone (ng/ml)	1.14±0.02	0.95±0.04*	0.82±0.01***	0.46±0.07***	0.36±0.04***

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ respectively as compared to control.

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Table: 4. Mean ± SEM of seminiferous tubule diameter (µm), tubular lumen diameter (µm), epithelial height (µm), area of seminiferous tubule (%) and interstitial space (%) of testis in control and extract treated groups.

Parameters	Extract Treatment (µg/mL)				
	Control	1	10	100	1000
Head length (µm)	163.50± 4.47	159.20±3.95	156.80±5.22	144.90±2.77**	142.90±3.30**
Tail length (µm)	26.40±2.61	27.50±3.04	31.50±2.15	39±1.87*	41±5.79*
DNA in head (%)	90.10±1.04	87.15±1.76	86.18±1.08	74.27±3.30***	69.82±4.81***
DNA in tail (%)	9.90±1.04	12.85±1.76	13.82±1.08	25.73±3.30***	30.18±4.81***
Tail moment (µm)	2.73±0.56	3.14±0.22	3.66±0.53	6.56±0.85***	5.94±0.95**

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ respectively as compared to control.

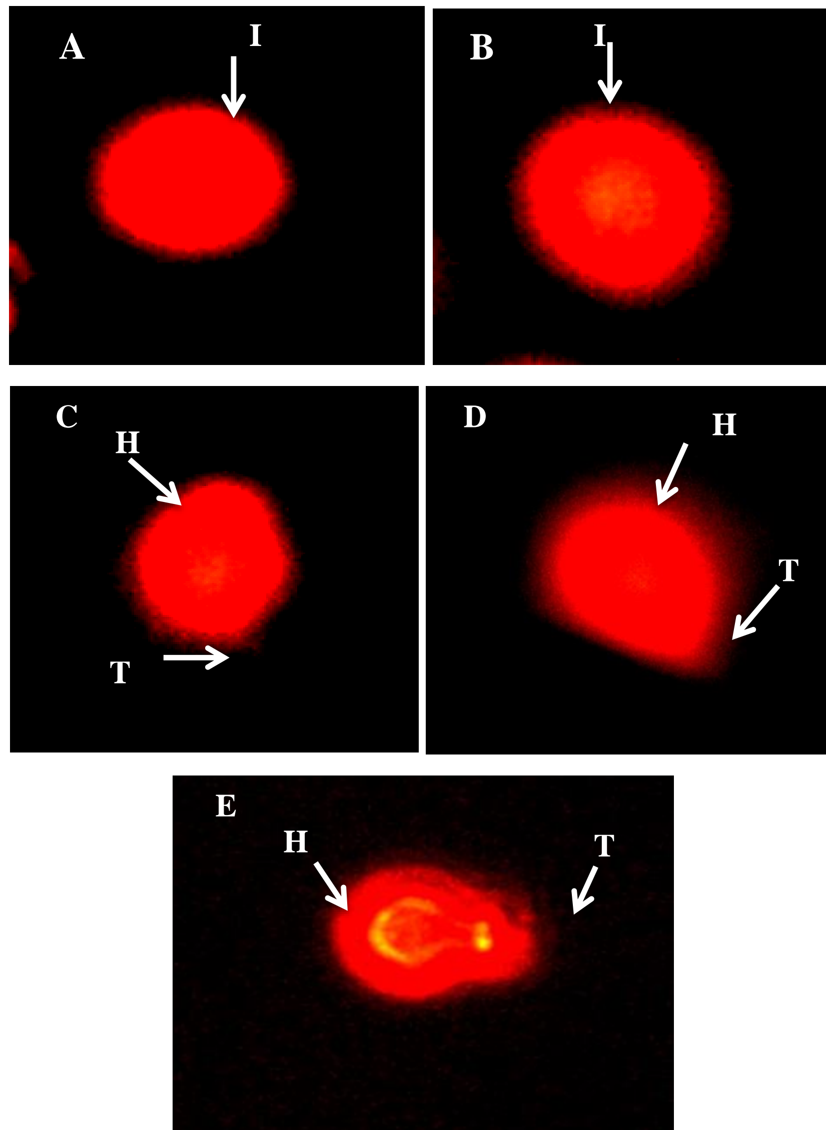

Figure 2: *In vitro* effect of methanolic leaf extract of *C. ambrosioides* on total length of chromatin dispersion in the sperm structure treated with (A) control (0 µg/mL), (B) 1 µg/mL, (C) 10 µg/mL, (D) 100 µg/mL and (E) 1000 µg/mL. 40 X. Head (H), Tail (T), Intact (I).

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