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Article

Pharmacological Impact of Chenopodium ambrosioides on Male Reproductive Health: Insights from Oxidative Stress, Hormonal Profiling and DNA Damage

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Abstract: Herbaceous plant species Chenopodium ambrosioides has natural compounds with anti-androgenic qualities and can be utilized as a contraceptive source. The goal of the current study was to investigate the antifertility activity of C. ambrosioides leaf extract in adult male rats using phytochemical analysis. Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant capacity of the methanolic extract were assessed. Additionally, GC-MS analysis was performed to closely examine the extract's bioactive phyto-constituents. An in vitro experimental approach was employed to evaluate the antifertility action by directly observing the impact of varying amounts of plant extract on the integrity of sperm DNA, the antioxidant state, and the release of testosterone in the testis. Results showed presence of adequate amount of total flavonoid and phenolic contents along with antioxidant potential. A GC-MS investigation of the methanolic leaf extract revealed that C. ambroisoides contains thirteen bioactive components. The maximum dose regimen (1000 μg/mL) treatment in the in vitro experiment showed significantly higher oxidative stress and decreased antioxidant activity. The rat sperm experience DNA damage as a result of the elevated ROS and lipid peroxidation concentration. Similarly, following two hours of incubation with all of the chosen dosages of C. ambrosioides leaf extract, a decrease in the levels of testosterone in the testicles was observed. According to the current study's findings, C. ambrosioides may affect male fertility by causing oxidative stress, hormonal imbalances, and sperm DNA integrity disruption in rat testicles exposed to greater extract concentrations.

Keywords: Chenopodium ambrosioides; GC-MS Sperm; DNA damage; Oxidative stress; Hormonal analysis

1. Introduction

The world population is growing at an exponential rate, which has an impact on the environment, health, and economic growth of developing and underdeveloped nations. There would be a greater need for effective actions in these circumstances. Many attempts have since been undertaken to reduce the birth rate through different strategies, which has resulted in a number of serious side effects include weight gain, hypertension, and hormone imbalances. Therefore, it is essential to swap out harmful chemicals for safe and efficient alternatives, such as plant-based contraceptives [1].

Throughout many years, people have used plants and herbs to control fertility. They can be used as a natural form of contraception with fewer adverse effects since they contain chemicals that have both androgenic and anti-androgenic qualities. It is difficult to develop a male contraception that is safe, effective, reversible, and doesn't affect libido or sexual activity. Numerous plants with terpenes, flavonoids, quinines, and tannins have been shown to have antifertility properties in the past. It is well known that plants in the Chenopodiaceae family are rich in alkaloids, flavonoids, phenols, and saponins [2].

The fragrant perennial shrub Chenopodium ambrosioides, also referred to as "Skhabotay (Kamasal Bhang)," is a member of the Chenopodiaceae family and is widely grown around the world. Though nothing is known about C. ambrosioides' toxicological characteristics, the plant has traditionally been utilized as a culinary condiment in traditional medicine. A rather high intake of C. ambrosioides essential oil has been linked to intoxication in both humans and

rats in a prior study [3]. The presence of terpenoidswhite., which have various pharmacological characteristics but also toxic features, is likely responsible for these harmful effects. Furthermore, C. ambrosioides possesses toxicity against a variety of insects and may find application as a botanical insecticide [4]. In a different investigation, an in vitro method using an aqueous seed extract of a closely related species, C. album, immobilized spermatozoa and damaged the sperm plasma membrane in rats and rabbits. Aqueous extract of C. ambrosioides has been demonstrated to have a deleterious effect on Drosophila melanogaster reproduction [5].

We have previously used adult male rats to assess the in vivo antifertility potential of C. ambrosioides. Consequently, based on earlier research.

The goal of the current study is to use an in vitro experimental approach to perform phytochemical analysis and antifertility assessment of C. ambrosioides leaf extract on testicular antioxidant status, testosterone production, and DNA integrity in rat spermatozoa, given the paucity of literature and knowledge along with its extensive and widespread traditional use.

2. MATERIALS AND METHODS

2.1. Plant collection and extract preparation

We collected fresh C. ambrosioides leaves from Abbottabad's farmed and agricultural lands. The plant was chosen due to its wide-ranging therapeutic applications in Asia and the location of its collection.

After removing the leaves from the stem, the plant was allowed to air dry, ground in a waring blender, and sieved. For seven days, leaf powder was immersed in 99.9% methanol (leaves to solvent ratio: 1:10). As per the previously published procedure outlined [6], the extract was filtered through Whattman filter paper and concentrated using a rotary evaporator (Model: Hei-VAP Heidolph, Germany). After being dried at room temperature, the filtrate mass was kept cold, at 4°C.

2.2. Phytochemical analysis Total flavonoid and Phenolic contents estimation

With a few minor adjustments, the previously published aluminum trichloride (AlCl3) colorimetric method was utilized to determine the total flavooids content. However, the Folin-Ciocalteu method—which Jagadish had previously described, albeit with a few minor adjustments—was used to determine the total phenolic contents [7].

2.3. Total antioxidant capacity determination

The phosphomolybdenum method was utilized to ascertain the extracts of plants' overall antioxidant capability. This approach reduces Mo (VI) to Mo (V), which results in the formation of a green-colored phosphate/Mo (V) complex [8].

2.4. Gas chromatography-mass spectrometry analysis (GCMS)

GCMS-QP5050 Shimadzu, Japan was used to do the GCMS analysis. The DB-5/RTX-MS capillary column, which had a length of 30 meters and a diameter of 0.25 millimeters, was composed of 95% dimethyl polysiloxane. With a linear velocity of 37.2 cm/sec and an injection volume of 1 μL, helium was utilized as the carrier gas, flowing at a rate of 1 ml/min. After injection, the column temperature was kept at 90°C for one minute in order to analyze the sample.

The temperature of the injector was raised to 200°C, increasing by 10°C each minute. After 15 minutes, the temperature was raised to 250°C at a rate of 10°C per minute. The injector was kept at 250°C, while the detector was kept at 300°C. A 70eV ionization energy was employed in the election ionization system. The sample was run for 60 minutes while the pressure was kept at 60.0 kPa. A scan rate covering a mass range of 35 to 600 amu was applied, with a cycle duration of 0.2 s [7,8].

2.5. Animals

The primate facility of Animal Sciences provided twenty-four adult male Sprague Dawley rats, ranging in age from 70 to 90 days. Animals were housed in well-ventilated rooms and arranged in stainless steel cages at random. Acclimatization was facilitated by a temperature of 26±1 °C and 10 / 14 h dark/light cycles. Water was accessible in plastic bottles, and the animals were fed normal lab-made food. The Animal Sciences Ethical Committee approved every experiment and protocol.

2.6. In vitro experiment

For this investigation, twenty-four mature male Sprague Dawley rats were employed. Moundipa's experimental design was created with a few changes. The range of doses was chosen in accordance with OECD guidelines and earlier in vitro studies employing plant extracts as advised because, to the best of my knowledge, no published in vitro study utilizing methanolic extracts of this plant has been reported [9]. For this investigation, five distinct dosages (0, 1, 10, 100, and 1000 μg/mL) were chosen. Plant extract stock solution was made in methanol and then combined with medium for cell culture. Less than 0.5% of the methanol content was retained in the prepared media.

2.7. Superoxidase dismutase assay (SOD)

Superoxidase dismutase activity was assessed using a method that has been previously published. Phenazinemethosulphate (18 uM, 0.1 ml), sodium pyrophosphate buffer (0.052 mM, 1.2 ml with pH 7.0), and 0.3 ml of homogenate were added to create a reaction mixture. NADH (0.2 ml, 780 uM) was added to initiate the reaction, and after 60 seconds, 1 ml of glacial acetic acid was added to end it. Changes in the color intensity of the chromogen produced were correlated with changes in the absorbance of the reaction mixture. Using a spectrophotometer, absorbance at a wavelength of 560 nm was recorded. Protein value was expressed in units/mg.

2.8. Peroxidase assay (POD)

In their respective investigations, Chance and Maehly (1955) had previously recommended methodologies for assessing peroxidase (POD) activity. A cuvette holding 1000μl of homogenate, 2.5 ml of PBS, 50 mM, pH = 5.0, 0.1 ml of guaiacol, 20 mM, and 0.3 ml of H_2O was used to prepare the reaction mixture. At 470 nm, absorbance was measured after a minute. POD was measured in milliunits per milligram of protein.

2.9. Catalase assay (CAT)

Catalase activity was measured using the previously published technique.Revisions to Chance and Maehly (1955). Supernatant (0.1 ml), H_2O (0.4 ml, 5.9 mM), and PBS (2.5 ml, 50 mM, pH 5.0) were added to the cuvette to create the reaction mixture. Using a spectrophotometer, the absorbance of the reaction mixture was measured at 240 nm after one minute. One unit of CAT activity was defined as an absorbance change of 0.01 units per minute.

2.10. Estimation of lipid peroxidation assay (TBARS)

Testicular homogenate's lipid peroxidation was calculated using the method described by [10]. PBS (0.58 ml, 0.1M pH 7.4), ascorbic acid (0.2 ml, 100mM), and ferric chloride (0.02ml, 100mM) were added to the test tube. Subsequently, 0.2 ml of homogenate was added to the mixture and allowed to incubate for an hour at 37°C. After the incubation period, 1.0 milliliter of trichloroacetic acid (10%) was added to stop the reaction. Subsequently, 1.0 milliliter of thiobarbituric acid (0.67%) was added to each tube, and it was once more incubated in boiling water for approximately half an hour before being transferred to crushed ice. For ten minutes, samples were centrifuged at 2500 g. The mixture's absorbance was measured at 535 nm in wavelength.

2.11. Estimation of Reactive Oxygen Species (ROS)

The approach previously disclosed [7] was utilized to assess the concentration of reactive oxygen species (ROS) in testicular tissue. H2O2 standards were made by dilutions, and 5 μL of standard and homogenate were added to a 96-well plate together with 140 μL of sodium acetate buffer (0.1 M, pH= 4.8). The plate was then allowed to incubate for five minutes at room temperature. After preparing a 1:25 solution, N, N-diethyl-para-phenylenediamine (DEPPD) and ferrous sulphate were incubated for 20 minutes in the dark. This solution was poured into a well and fully stirred to make 100 μL. Using a microplate reader set to record at 505 nm for 90 s at 15 s intervals, absorbance was measured. The concentration of hydrogen peroxide in the sample was equal to one unit of ROS.

2.12. Hormonal Analysis

By following the manufacturer's instructions, the amount of testosterone in testicular homogenate was quantitatively evaluated using Enzyme Linked Immuno Sorbant Assay (ELISA) kits (Biocheck Inc., USA).

3. Statistical analysis

Dunnet's multiple comparison test was performed after one way analysis of variance (ANOVA) was used to assess the data. Graph Pad Prism 5 software was used to compare different dosing groups with the control group. A significance threshold of $p<0.05$ was used.

4. RESULTS

4.1. 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.

4.2. Antioxidant potential Estimation

The DPPH test was used to measure the free radical scavenging activity of the methanolic leaf extract of C. ambrosioides. The IC_{50} value was computed in order to assess the outcomes. As indicated in table 1, it was found that methanolic leaf extract has free radical scavenging activity with an inhibitory concentration (IC_{50} value) of 56 μg/mL.

4.3. Gas chromatography-Mass spectrometry (GC-MS) analysis

Table 2 provides information on the various chemicals found in the methanolic leaf extract of C. ambrosioides, whereas figure 2 displays the GC-MS chromatogram.

These substances range in nature and include amino acids, phytol, and methyl ester of fatty acids. With a percent area of 9.19%, arginine was the most common compound in the study. It was 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%) in addition to minor constituents.

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

Rat testicular tissue's biochemical and hormonal profile was ascertained during a two-hour incubation period with varying quantities of C. ambrosioides' methanolic leaf extract (Table 3). There was a decline in catalase activity in the high dose treated group that was dose dependent. In comparison to the control, there was a substantial reduction in the 10 μg/mL and 1000 μg/mL dose treatment groups ($p<0.01$ and $p<0.01$, respectively). Similarly, an in vitro experimental technique was used to determine the SOD and POD efficacy of a methanolic leaf extract of C. ambrosioides on the reproductive system of male rats.

The results of the phytochemical study showed that the methanolic extract contained a significant amount of total flavonoid contents (measured in quercetin equivalent) and total phenolic contents (measured in gallic acid). These findings were consistent with earlier research that revealed substantial levels of phenols and flavonoids in the crude extract of H. nepalensis and its fractions [5]. Both flavonoids and phenols have a significant impact on stabilizing lipid oxidation and are closely linked to the biological system's antioxidant activity [3]. One inexpensive technique for evaluating antioxidant capacity is to measure the amount of free radical DPPH scavenging activity. This procedure depends on the acceptance of an electron by antioxidants [3,6], which transforms purple-colored DPPH into yellow-colored 2,2-diphenyl-1-picryl-hydrazyl.

The phytochemical components of the C. ambosioides methanolic leaf extract were examined using GC-MS in the current investigation. A number of known chemicals, including lipids, amino acids, and phytol, have been found to be present in the methanolic leaf extract of C. ambrosioides. Several of the chemicals have anti-inflammatory, antibacterial, and anticancer properties. Fatty acids and lipids contribute to the production of systemic oxidative stress, which is known to be harmful to sperm quality and linked to infertility, validating the current study's claim.

The disruption of cellular homeostasis, dysregulation of the signaling network, and damage to proteins, nucleic acids, and lipids resulting in genomic instability are all well-known effects of oxidative stress caused by ROS and RNS. Overproduction of ROS and RNS can result in a number of illnesses, such as rheumatoid arthritis, diabetes, hypertension, neurodegeneration, cardiovascular disorders, and atherosclerosis. Due to the presence of unsaturated fatty acids and reactive oxygen species, which cause lipid peroxidation, tests are known to be more sensitive to oxidative stress [8]. The results of this investigation demonstrated that testis exposed to varying concentrations of C. ambrosioides extract

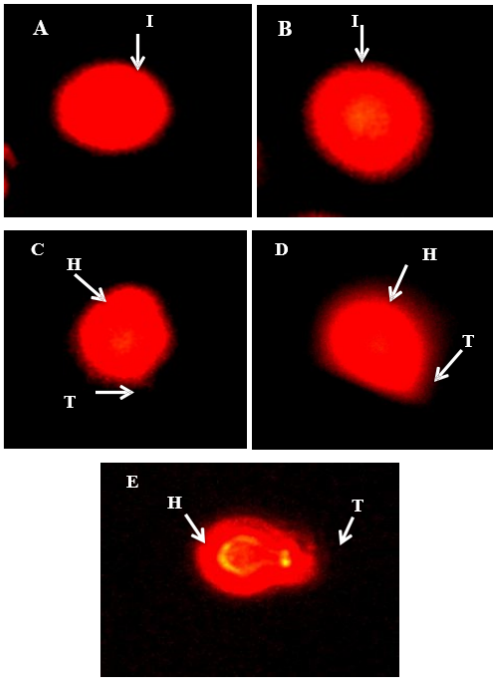

Figure 2. In vitro impact of C. ambrosioides leaf extract on sperm chromatin dispersion length at varying concentrations

experienced an increase in TBARS and ROS and a decrease in testicular antioxidant levels (SOD, POD, and CAT). On the other hand, tissues cultured with high dose regimens showed a greater presence of this oxidative stress. This extract from a plant caused oxidative damage [2].

The methanolic extract of C. ambrosioides affects sperm DNA following exposure at higher dosages (100 and 1000 μg/mL), according to the comet assay results in the current study. Numerous in vitro and in vivo investigations have previously demonstrated the connection between oxidative stress and sperm DNA damage and testicular dysfunction [3,5]. Similar results were previously reported, wherein fenugreek seed administration to mice reduced fertility in a dose-dependent manner by altering sperm parameters and producing abnormal sperms with DNA damage, suggesting a connection between oxidative stress and DNA damage leading to male mouse infertility [10].

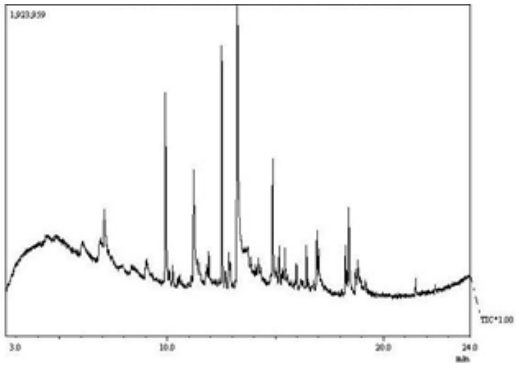

Figure 1. GC/MS chromatogram of methanolic leaf extract of C. ambrosioides

In the current investigation, testicular testosterone concentrations were lowered by incubating testicular tissues with C. ambrosioides methanolic leaf extract. Regulating fertility and spermatogenesis require normal amounts of testosterone in the blood. Testicular tissue under increased oxidative stress may interfere with steroidogenesis by lowering hormone levels through Leydig cells [5].

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 IC_{50} 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

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 #
7.093	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

	Extract Treatment (μg/mL)				
Parameters					
	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)	22.37±2.38	15.12±2.32	19.68±2.38	16.71±3.28	14.63±1.98*
POD (nmole)	14.54±1.08	12.31±1.27	10.55±1.54	12.56±1.28	6.84±1.48*
TBARS (nM/mg tissue)	17.32±1.81	18.68±1.72	20.67±3.07	22.01±3.04	25.37±2.92
ROS (U/g tissue)	15.51±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***

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

	Extract Treatment (μg/mL)				
Parameters					
	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**

5. CONCLUSION

According to the results of this investigation, C. ambosioides leaf extract in methanolic form has a significant amount of flavonoids and phenolic compounds that have the potential to be antioxidants. GC-MS study revealed that it contains a range of components that may be utilized as innovative scaffolds in the investigation of contraceptive drug discovery. The present study offers a foundation and guidance for future pharmacological explorations of the examined plant. Additionally, the outcomes of an in vitro investigation showed that C. ambosioides methanolic leaf extract has the capacity to cause oxidative stress in testicular tissue, which is linked to a decrease in testicular testosterone and damage to sperm DNA. 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.

Author Contributions: All authors contributed equally to the writing of this paper. All authors read and approved the final manuscript.

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