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EVALUATION OF THE THERAPEUTIC EFFECTS OF *Cucurbita pepo* L. LEAF AND SEED METHANOLIC EXTRACTS ON INDUCED INFERTILITY IN FEMALE ALBINO RATS

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ABSTRACT

Infertility is now one of the major health challenges among all ages. Excessive production of ROS and inflammation is believed to be one of the main etiology. This study aimed to determine the therapeutic effects of *Cucurbita pepo* L. leaf and seed methanolic extracts on induced infertility in female albino rats. Thirty albino rats (both sexes) were grouped into 6 groups of 5 rats each. The male rats were used for mating. Group1 (Normal) and group 2 (Untreated), group 3,4, 5 and 6 were administered 20mg/kg of $AlCl_3$ and treated with 200mg/kg seed, 200mg/kg leaf, 400mg/kg in (1:1 mixture) of seed & leaf extracts and standard drugs for 8weeks respectively. The phytochemical & biochemical evaluation was carried out using spectrophotometric and the hormonal assay with ELISA techniques. The phytochemical results revealed the presence of alkaloids, steroids, flavonoids, to be 37.15 ± 0.30, 28.42 ± 1.17, 23.62 ± 0.57 respectively. Both the extracts showed a significant antioxidant and anti-inflammatory activity. This increased significantly ($p<0.05$) the serum concentration of SOD and Gpx with significant ($p< 0.05$) decrease in the serum level of MDA in all the treatment compared to group 2. The result also exhibited an excellent potency against inflammation due to the significant decrease ($p<0.05$) in the serum level of IL-6 in the treatment group compared to group 2 with 226.89 ± 3.45. The hormonal results also revealed effects on some female hormones compared to group 1 and 2. This showed that the extracts have protective effect on the $AlCl_3$ damage.

Keywords: *Cucurbita pepo,* $AlCl_3$, Infertility, Antioxidants

INTRODUCTION

Reproduction is indeed indispensable for ensuring the continuity of human populations. Recently, reports indicate that one in six couples worldwide experiences difficulty in becoming pregnant and seek for medical assistance (WHO, 2023). According to latest World Health Organization (WHO, 2023) statistics, infertility is the fifth highest serious condition worldwide with negative impact on self-esteem which remain a high social burden on women than men (Deshpande & Zayed *et al*., 2019, 2020).

WHO (2023), also described infertility as a disorder that affect both male and female reproductive system manifested by inability to conceive or carry a pregnancy to full term. It is either classified as primary, if conception has never been achieved or secondary if conception has been experienced before and unable to conceive for the second time even after having regular unprotected intercourse for one or more years (Poddar, 2014). Infertility is a multifactorial stressor with approximately 20-25 % of infertility cases due to male infertility factors, 20-58% are due to female infertility factors, and 25-40% are due to combined problems in both parties (Smeenk *et al*., 2019). In 10 – 17 % cases no cause is found which is termed unexplained or idiopathic types of infertility (Smeenk *et al*., 2019).

The most common cause of female infertility is ovulatory problem which generally manifest itself by sparse or absence of menstrual periods (NHS, 2017). Other common causes of female infertility are ovarian problems (such as polycystic ovarian syndrome). Generally, the leading reason why women present to fertility clinic is due to an ovulatory infertility (Balen *et al.*, 2006). Tubal blockage due to pelvic inflammatory disease, uterine problems, previous tubal ligation, endometriosis and advanced maternal age. Mostly, women are more fertile within the age of 24 and diminishes after 30 with pregnancy occurring rarely after age 50 (Pastore *et al*., 2018). This is because millions of oocytes may be present at birth, but decline over time and approximately 300 to 500 oocytes will be ovulated during reproductive life span and almost zero at the age of 50 (Pastore *et al*., 2018).

Despite the rate of infertility, prevention, diagnosis and treatment of infertility specifically assisted reproductive technology such as IVF remain unfunded, un affordable and inaccessible to many individual due to stress (being financial, economic, psychological stress) and social stigma (WHO, 2023). Functional food has been shown to be an alternative treatment for infertility and many several anomalies. Pumpkin plant is an edible fruit and represent a quick, easy, and readily available source of micronutrient and functional compounds that provide numerous health benefits (Chandrasekhar *et al.*, 2021). Cucurbits are one of the major and diverse groups of plant families that are cultivated, as the seeds of these plants exhibit a wide array of therapeutic activity.

Figure 1: Pumpkin Leaf, Fruit and Seed

The interest in using functional plants to ameliorate fertility problems and several health ailments may be due to the belief that functional foods have little or no side effect, boost immunity, its affordable, available and nutritious. Pumpkin plants has been recognized as one of the functional plants and this calls for the search for its therapeutic effects especially the Methanolic extracts of pumpkin seed and leaf.

Statement of the Research Problem

The disorders in the female reproductive organs that lead to infertility is now a crisis amongst young ages which has negative impacts on several life dimensions such as violence, divorce, social stigma, emotional stress, depression, anxiety and low self-esteem (Gore *et al*., 2015).

Infertility might be due to genetics, lifestyle changes factors such as aging, medication, diet, obesity and environmental factors such as air pollution, infections, radiation, and toxic chemicals sources (WHO, 2024). *All these factors can significantly stimulate the excessive release of reactive oxygen species that can lead to oxidative stress and inflammation.* It is estimated that the prevalence will increase in the subsequent years (WHO, 2024). One of the major way to mitigates this challenges is the use of fertility medications. Functional plants have been known to remediates the problem of infertility and several health issue worldwide. This research hypothesizes that Aluminum trichloride can affects female rat's reproductive functions negatively and seeks to evaluate the ameliorative effect of pumpkin (*Cucurbita pepo* L) leaf and seed against the disorder.

Aim and Objectives

The aim of this research is to determine the therapeutic effects of *Cucurbita pepo* leaf and seed extracts on $AlCl_3$ induced infertility in female albino rats.

The specific objectives are to determine:

i. The qualitative and quantitative phytochemical components of pumpkin leaf and seeds extracts.
ii. The effect of the methanolic extracts of pumpkin seed and leaf on oxidative status levels.
iii. The effect of the methanolic extracts on fertility hormones (FSH, LH, progesterone – P4).
iv. The effect of the methanolic extracts of *Cucurbita pepo* L. leaf and seed on the pro-inflammatory cytokines (IL-6).

Significance of the Study

Infertility, particularly when associated with oxidative stress and inflammatory responses, remains a pressing global health concern with wide-ranging psychological, social, and economic consequences. Existing therapeutic approaches often involve synthetic drugs or assisted reproductive technologies, which are not only expensive and sometimes inaccessible but may also result in undesirable side effects. This study is therefore significant because it explores the therapeutic potential of *Cucurbita pepo* (pumpkin) leaves and seeds, which are widely consumed as food and have long been used in traditional medicine, as a safe, affordable, and natural alternative for the management of infertility.

Scope of the Study

The scope of this research is limited to the investigation of the therapeutic effects of *Cucurbita pepo* leaf and seed extracts on aluminum chloride ($AlCl_3$) induced infertility in female albino rats.

MATERIALS AND METHODS

Materials

The materials used for this study are listed below

Plant

Cucurbita pepo (Pumpkin) leaves and seeds.

Chemicals/Reagents

All chemicals/reagent used were analytical graded and include aluminum chloride anhydrous ($AlCl_3$) (Xilong chemicals), methanol (ENAT400IU), natural vitamin E, Folin-Ciocalteu reagent, garlic acid, phosphate buffer, sodium azide, hydrogen peroxide, reduced glutathione (GSH), trichloroacetic Acid (TCA), thiobarbituric acid (TBA) and ELISA Kits (Booster Biological Technology Co Ltd, California, U.S.A).

Equipment/Instruments

Spectrophometer (UV, Scitek Global Co. Ltd), Bench centrifuge (614B), Refrigerator, Electric blender (Sonifer) Rotary evaporator (RV 10 Digital V), Water Bath (LX603NDB), weighing balance (Shanghai, model Yp6001), Soxhlet extractors apparatus (Eisco Labs)

Sample Collection and Identification

Freshly harvested fruit and leaves of *Cucurbita pepo* was purchased from Dokari Farm, Keffi and were taken for identification and authentication at Ahmadu Bello University Zaria where voucher the number was obtained from the ABU Herbarium (ABUH02873) Department of Botany. The leaves were washed and air dried for three (3) weeks, while the fruit was open to obtain the seeds which was also washed, air dried for the same duration and packaged well for further analysis.

Experimental Animals

Thirty (30) healthy albino rats (both sexes) were purchased from Ahmadu Bello University, Department of Zoology. The male rats were introduced 7 days for mating purposes only. The animals were housed separately in a well ventilated iron cage for three (3) weeks, acclimatization, fed with Chikun broiler finisher pellet feed (obtained from prime care consults Keffi) and clean water prior to the commencement of the experiment. The animals were maintained at room temperature with a 12hr – 12hr dark- light cycle. The body weight of each rats was duly checked before and after acclimatization periods to determine possible weight changes during experimentation.

Ethical Clearance Approval

Ethical Clearance approval was collected from NSUK Animal Care and Use Research Ethics Committee (NSUK-ACUREC). All the experimental steps were done according to the ethical rules of NSUK-ACUREC, Nasarawa State University, Keffi. See the appendix for the copy of ethical clearance (NSUK- ACUREC/BCH/24/11).

Methods

Methods of Extraction

The *Cucurbita pepo* leaves and seeds collected were dried and grinded to pass 0.5 mm sieve to provide a greater surface area and weighed according to James *et al.* (2014). An average of 50g of the dried powder was filled in the porous cellulose thimble and subjected to Soxhlet extraction using 98% methanol for 12hours at 75°C, followed by filtration through a Whatman No.1 filter paper. The methanol Extract obtain was concentrated to dryness at 45°C using a rotary evaporator under reduced pressure and the extract was weighed and then stored at Extracts were stored at −20°C until use.

Experimental Design

Twenty-four (24) female albino rats were grouped into six (6) with a male in each group, of which all groups were administered orally with 20mg of $AlCl_3$ dissolved in water, per body weight except the group one animals, which are the normal control.

The groups were labeled as follows:

Group 1: Normal feed + water (Normal)

Group 2: $AlCl_3$ in water + feed and Water (Induced, untreated group)

Group 3: $AlCl_3$ +200mg/kg (Seed extract)

Group 4: $AlCl_3$ + 200mg/kg (Leaf extract)

Group 5: $AlCl_3$ + 400mg/kg (200mg/kg Seed + 200mg/kg Leaf extracts) in 1:1 mixture

Group 6: $AlCl_3$ + 400IU Vitamin E (Standard drug)

Collection of Sample

$AlCl_3$ was administered daily for eight (8) weeks, extracts were given concurrently starting from week 1 with critical observation, after which the animals were sacrificed in accordance with the guidelines of the European Convention for the protection of Vertebrate Animals and other scientific purposes ETS-123 (European Treaty Series, 2005). Whole blood sample of each rats was collected using sterile needle and syringe into a plain tube. The blood samples were allowed to stand and clot at room temperature for two hours before centrifugation at 1000rpm for 10minutes using bench centrifuge to separate the serum from the cell. Another sterilize plain tube was labeled and used to collects aliquots of serum and stored in the refrigerator for various biochemical analysis.

Phytochemical Screening

Qualitative and quantitative analysis of phytochemicals present in the study sample were carried out using standard procedures as described below.

Qualitative Phytochemical Screening

Pumpkin leaves extract was screened for the presence of different classes of phytochemicals *such as* alkaloids, flavonoids, glycosides, phenols, saponins, steroids, tannins terpenoids, cardiac glycoside.

Test for Alkaloids

To 5 ml of extract, 2 ml of HCl and 1 ml of Dragendroff's reagent was added. An orange or red precipitate was used as an indication of positive results for alkaloids (*Chaudhary et al.,2010).*

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Test for Flavonoids

Exactly 1.0 ml of extract was taken and 10% of lead acetate was added. The formation of yellow precipitate indicated positive inference for flavonoids. (Vishnu *et al.,* 2019)

Test for Glycosides (Born Trager's Test)

To 2 ml of the extract, 3 ml of chloroform was added and shaken. The chloroform layer was separated and 10% ammonia solution was added. The formation of pink color was used as an indication for the presence of glycosides (*Chaudhary et al.,2010)*.

Test for Phenol

To about 5 ml of extract, 3 ml of 10% acetate solution was added and mixed gently. The formation of bulky white precipitate indicated the presence of phenol with little modification (Vishnu *et al.,* 2019).

Test for Saponins

Exactly 0.5mg of extract with few drops of distilled water was vigorously shaken. The formation of frothing was used as an indication for the presence of saponins (*Evans., 2009).*

Test for Steroids

To 2ml of extract, 2ml of chloroform and 2ml of concentrated H_2SO_4 was added. The appearance of red color and yellowish-green fluorescence indicated the presence of steroids with little modification (*Evans., 2009).*

Test for Tannins

To 5 ml of extract, few drops of 5% ferric chloride solution was added. The production of dark green color indicated the presence of tannins (Vishnu *et al.,* 2019).

Test for Terpenoids (Salkowski Test)

To 3ml of the extract, 1ml of chloroform and 1.5 ml of conc. H_2SO_4 was added along the sides of the tube. The reddish-brown color in the interface was considered positive for the presence of terpenoids (*Chaudhary et al.,2010).*

Quantitative Phytochemical Screening of the Plant Extract

Quantitative phytochemical screening was carried out according to standard operating procedures with modifications.

Estimation of Total Alkaloids Content

The total alkaloids content was determined according to the method described by Raheleh *et al.* (2013) with little modification. Exactly 100 ppm solution of atropine (1mg in 10 ml of distilled water) was used as standard. From this stock solution, exactly 0.5, 1.0, 1.5, 2.0 and 2.5 ml of atropine solutions was transferred to five different separating funnels. To each separating funnel, 5 ml of phosphate buffer (pH 4.7) and 5 ml Bromocresol green (BCG) solution was added and mix vigorously. The formed complex mixture was extracted with chloroform (5ml). The chloroform fraction was collected in a 10 ml volumetric flask and made up the volume with chloroform. Absorption at a wavelength of 470nm of each flask was measured and a calibration graph was drawn. For the presentation of sample, the plant extract (1mg/ml) was dissolved in 2N HCl and then filtered. The pH of the extract was adjusted to neutral with 0.1N NaOH. Exactly 1ml of this solution was transferred to a separating funnel and to the mixture of Bromocresol green solution along with 5ml of phosphate buffer was added and mixed properly. The mixture was extracted further with 5ml of phosphate chloroform and transferred to 10ml volumetric flask and make the volume with chloroform. The absorbance of the complex in chloroform was measured at 470nm.

Phenolic Content

The total phenolic content was determined according to the method describe by Mauryan and Singh (2010). This is basically a color reaction that produces a blue color which is measured spectrophotometrically. Folin Ciocalteu reagent was used to evaluate the amount of total phenolic content and gallic acid was used as standard express as mg/g garlic acid equivalent (GAE). The concentration of 1 mg/ml of plant extract was prepared in methanol and 0.5 ml of the sample was placed on a test tube which was mixed with 2.5ml of 10-fold diluted Folin-Ciocalteu Reagent. The mixture was added to 2ml of 7.5% of sodium bicarbonate. The tubes were covered with para-film and allowed to stand for 30mins at room temperature before taking the absorbance at 760nm.

Estimation of Tannins

Determination of total tannins content was evaluated by using Folin-Ciocalteu's reagent (Rajeev *et al.,* 2012). Plant extract (10mg) was dissolved in 10 ml of distilled water to make final concentration of 1 mg/ 1ml. Different aliquots of garlic acid (0.5, 0.4, 0.3, 0.2 and 0.1 mg) was dissolved in 1 ml of distilled water to make the final concentrations 100, 80, 60, 40 and 20 ugh/ml. After preparing 3.5% v/v sodium carbonate solution, 0.1ml garlic acid solution of every concentration (100-120 mg/L) was taken into different volumetric flask. Then 7.5 ml of distilled water and 0.5 ml FC reagent was added to it. After 5mins, 1 ml of 3.5% sodium carbonate solution and 10 ml distilled water was added to that flask. After 30mins, UV absorbance was determined at 725nm. Total tannin content of the extract was measured using garlic acid standard calibration curve and stated as mg garlic acid equivalent, GAE/100g of dried plant extract.

Flavonoids Content

The total flavonoids content was determined according to the method describe by Zengnin *et al.* (2011). A standard solution (10-50 µgh/ml) of rutin was added to 75 µl of sodium nitrite ($NaNO_2$, 5%) solution and mixed properly. After 5-6 minutes, 0.5 ml of aluminium chloride ($AlCl_3$, 100g/l) was added. 0.5 ml of sodium hydroxide (NaOH, 4%) was added after 5 minutes. The final volume was kept at 2.5ml with distilled water and thoroughly mixed. The absorbance of the mixture was determined at 510nm against the same mixture without the sample as blank. Total flavonoids content was expressed as mg rutin/g dry weight (mg/g DW) through the calibration curve of rutin. All samples were analyzed in triplicates.

Estimation of Total Terpenoids

Total terpenoids was determined by the method of Thakur, *et al*., (2019). In the test tube containing 200 µL of extract (1mg/ml), 1.5ml of chloroform was added. The sample was vortexed thoroughly and brought to rest for 3mins and then 100 µL of conc. Sulphuric acid was added to the test-tube and was incubated at room temperature for 1.5-2 hours in the dark. At the end of the incubation time, reddish brown precipitation was formed in each assay tubes. All the supernatant was carefully removed from the reaction mixture without disturbing the precipitation. Then 1.5ml of 95% (v/v) methanol was added and vortexed thoroughly until all the precipitation dissolves in methanol completely. The sample was transferred from assay tubes to colorimetric cuvette (95% [v/v] methanol was used as blank) to read the absorbance at 538nm by using UV-Vis Spectrophotometer. Quantification was based on the standard curve of linalool. All tests were carried in triplicates and results were expressed as linalool equivalent (µg of linalool/mg extracts).

Determination of Cardiac Glycosides

Exactly 10ml of the extract was pipetted into a 250ml conical flask. 50ml chloroform was added and shaken on vortex mixer for 1 hour. The mixture was filtered into 100ml conical flask. Pyridine (10ml) and 2ml of 29% sodium nitroprusside were added and shaken thoroughly for 10mins. Thereafter, 3ml of 20% NaOH was added to develop a brownish and yellow color. Glycosides standard (Digitoxin) was used at concentration range from 0-50mg/ml from stock solution and the absorbance was read at 510nm. The procedure described by Sofowora (1999) was used.

Determination of Total Saponins

The total saponins content was determined by spectrophotometry as described by Uematsu *et al.* (2000). To 2ml of each extract, 1ml of reagent A (p-anisaldehyde 0.5 in ethyl acetate 99.5) and 1ml of reagent B (H_2SO_4 50% in ethyl acetate 50%) was mixed and homogenized in a vortex. The methanol extracts of *C. pepo* and standard (Digitoxin) was dissolved in 2ml ethyl acetate. Then, 1ml of reagent A and 1ml of reagent B was added. The mixture was stirred and incubated at 60°C for 10mins in a water bath. The solution was cooled at room temperature for 10mins and the absorbance of the color developed solution was recorded at 430nm. Ethyl acetate was used as a control for the measurements of absorbance. Solution containing 75-175 µg standard (digitoxin) in 2ml ethyl acetate was used to obtain a calibration curve. The total saponins was determined from the calibration curve and expressed as milligrams of digitoxin equivalent pre grams of extract (mgDE/g).

Determination of Total Glycosides Content (TGC).

Balet's reagent colorimetric method was used for the determination of the total glycosides content of the extracts as previously reported by Tonight *et al.* (2016) with modification. The extract (1mg/ml) was dissolved with 6ml of distilled water and 1ml of 12.5% (w/v) lead acetate solution was added. The mixture was made up to 10ml with distilled water and filtered. The filtrate (5ml) was transferred to a volumetric flask, 1ml of 4.77% (w/v) Na_2HPO_4 solution was added and made up to 10ml with distilled water and filtered. Baljet's reagent (10ml) was added to 1ml of the clear filtrate and the mixture was allowed to stand for 1hour and diluted with 20ml of distilled water. The absorbance of the mixture was read against the blank at 495nm with a UV-Vis Spectrophotometer. The total glycosides content was determined from the calibration curve and expressed as milligrams of digitoxin equivalent per gram of extracts (mgDE/g).

Primary Enzymatic Antioxidant Activity Estimation

Estimation of Superoxide Dismutase Activity

The SOD activity was determined using the method of Kakkar *et al*. (1984).

Principle

Superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating system and catalyzes the inhibition of Nitroblue tetrazolium (NBT) reduction, the extent of which can be assayed spectrophotometrically.

Procedure

The incubation medium contained, a final volume of 3.0ml, 50mM potassium phosphate buffer (pH 7.8), 45M methionine, 5.3mM riboflavin, 84M NBT and 20M potassium cyanide. The amount of homogenate added to this medium was kept below one unit of enzyme to ensure sufficient accuracy.

The tubes were placed in an aluminum foil-lined box maintained at 25°C and equipped with 15W fluorescent lamps. Reduced NBT was measured spectrophotometrically at 600nm after exposure to light for 10 minutes. The maximum reduction was evaluated in the absence of the enzyme. One unit of enzyme activity was defined as the amount of enzyme giving a 50% inhibition of the reduction of NBT. The values were calculated as unit/mg protein.

Determination of Glutathione Peroxidase (GPx) Activity

The GPx activity was measured using the method of Ellman, (1959).

Principle

Glutathione peroxidase catalyzes the following reaction:

$$2GSH + H_2O_2 \xrightarrow{\text{Se-GPx}} GSSH + 2H_2O$$

Glutathione was measured by its reaction with DTNB to give a compound that absorbs at 412nm.

Procedure

To 0.4ml of buffer, 0.2ml of EDTA, 0.1ml of sodium azide and 0.2ml of reduced glutathione, 0.1ml of hydrogen peroxide were added to two test tubes labelled as test and control. To the test, 0.2ml of sample was added while to the control, 0.2ml of water was added. The contents were mixed well and incubated at 37°C for 10 min, the reaction was arrested with the addition of 0.5ml of 10% TCA. To determine the glutathione content, 1.0ml of supernatant was removed by centrifugation, to that 3.0ml of buffer and 0.5ml of Ellman's reagent. The color formed was read at 412nm.

Standards in the range of 40-200μg was taken and treated in the similar manner. The activity was expressed in term of μg of glutathione consumed/min/mg protein.

Estimation Malondialdehyde (MDA)

Lipid peroxidation was estimated by measuring spectrophotometerically the level of the lipid peroxidation product, malondialdehhyde (MDA) as described by Wallin *et al.* (1993).

Principle

Lipid degradation occurs forming such products as malondialdehyde (from fatty acids with two or more double bonds), ethane and pentane (from the n – terminal carbons of 3 and 6 fatty acids, respectively) malondialdehhyde reacts with thiobarbituric acid to form a red or pink colored complex which in acid solution absorbs maximally at 532nm.

$$MDA + 2TBA \longrightarrow MDA\text{-}TBA \text{ adduct} + 2H_2O$$

Procedure

Into two test tubes labeled test, serum and blank were added 10μL of serum, and 10μL distilled water respectively. Then, 0.5ml of 25 % TCA (Trichloro Acetic Acid) and 0.5 ml of 1 % TBA (thiobarbituric acid) in 0.3 % NaOH were added. The mixture was boiled for 40 minutes in a water bath and cooled in cold water. Then, 0.1ml of 20 % sodium dodecyl sulfate (SDS) was added to the solution and mixed properly. The absorbance was taken at wavelengths of 532nm and 600nm against a blank.

$$MDA = \frac{Abs_1 - Abs_2}{0.00693}$$

Biochemical Analysis of Female Steroid Hormones

Serum levels of progesterone, FSH, LH, was done using Enzyme Linked Immunosorbent Assay (ELISA) as described by Luisina Ongaro *et al*. (2021)

Procedure

Exactly 0.05ml of both samples was pipetted and mixed with serum and standard drug into assigned well. Also, 0.10ml of FSH-Enzyme reagent was added to all the wells and the microplate gently swirled for 20 seconds to mix. This was then covered and incubated for 60 minutes at room temperature.

The content of the microplate was discarded by decantation and the plate was blotted dry by using absorbent paper. Thereafter, 350ml of wash buffer was added, decanted and aspirated. This process was repeated twice for a total of 3 washes. Exactly 0.10ml of working substrate solution was then added to all the wells and incubated at room temperature for 15 minutes. Stock solution of 0.50ml was added to each well, shaken gently for 20 seconds. The absorbance was in each well was read at 450nm.

Calculation

$$\frac{\text{Absorbance of Samples}}{\text{Absorbance of Standard}} \text{ x Concentration of Standard}$$

Estimation of Interleukin-6 (IL-6)

Interleukin-6 (IL–6) levels was determined using ELISA techniques as described by Fevziye *et al*. (2021).

Procedure

Exactly 100µL of each of the sample, blank and sample was pipetted to each of the well. The plate was then covered with sealer and incubated for 90 minutes at 37^{O}C. The liquid of each of the well was removed and not wash immediately, and about 100µL of Biotinylated was added. The solution from each well was decanted and 350µL of wash buffer was added to each well. Soak for 2 minutes and the solution from each well was decanted and pat it dry against clean absorbent paper. Repeat 3 times using wash buffer.

Exactly 100µL of HRP conjugate working solution was added to each well, covered with the plate sealer and Incubated for 30 minutes at 37^{O}C. The solution from each well was decanted and the wash was repeated for 5times. Also 90µL of substrate reagent was added, cover with the plate sealer. Incubated for 15 minutes at 37^{0}C. Equally, 50µl of the stop solution was added to each well. The optical density of each of the well was determined with microplate reader at 450 nm.

Calculation

$$\frac{\text{Absorbance of Samples}}{\text{Absorbance of Standard}} \text{ x Concentration of Standard}$$

Statistical Analysis

The results obtained were analyzed using One- way Analysis of Variance (ANOVA) in IBM SPSS package version 26 and the results presented as Mean ± Standard Deviations. Multiple group comparisons were performed using Duncan's test at P<0.05 level of significant.

RESULTS AND DISCUSSION

Qualitative Phytochemical Composition of the Leaf Methanolic Extract of *C. pepo*

The results of the qualitative phytochemical analysis of the leaf extract of *C. pepo* is as shown in Table 4.1. It releveled the presence of alkaloids, steroids, flavonoids, phenol, saponins, tannins cardiac glycosides and glycosides, while terpenoids were absent.

Table 1: Qualitative Phytochemical Composition of the *C. Pepo* L. Leaf Methanolic Extract

Phytochemical	Composition
Phenols	+
Tannins	+
Saponins	+
Alkaloids	+
Steroids	+

Phytochemical	Composition
Cardiac glycosides	+
Glycosides	+
Flavonoids	+
Terpenoids	–

+ = Presence – = Absence

Quantitative Phytochemical Composition of the Leaf and Seed Methanolic Extracts *of C. Pepo* L.

The quantitative phytochemical screening of the leaf and seed extracts of *C. pepo* is presented in Table 4.2 indicating varying concentrations of phenols, flavonoids, alkaloids, and tannins amongst others. From the results observed, alkaloids have a concentration of 37.15 ± 0.30 mg/g while glycosides concentration is 4.76 ± 0.61 mg/g in the leaf extract. Results of the seed extract on the other hand revealed terpenoids has the concentration of 25.79 ± 0.62 mg/g, while tannins concentration is 11.76 ± 0.31 mg/g.

Table 2: Quantitative Phytochemical Composition of the *C. Pepo* Leaf and Seeds Methanolic Extracts

Phytochemicals	Extracts (mg/g)	
	Leaves	**Seeds**
Cardiac Glycosides	6.10 ± 0.62	Not detected
Glycosides	4.76 ± 0.61	Not detected
Flavonoids	23.62 ± 0.57	13.60 ± 1.19
Alkaloids	37.15 ± 0.30	Not detected
Tannins	10.33 ± 1.50	11.76 ± 0.31
Steroids	28.42 ± 1.17	Not detected
Saponins	13.07 ± 0.64	17.77 ± 0.12
Phenols	17.99 ± 1.47	15.03 ± 0.57
Terpenoids	Not detected	25.79 ± 0.62

Results are Expressed as Means ± Standard Deviation of Repetitive Values (n = 5) in mg/g Dry Weight.

Effect of Pumpkin Leaf and Seed Methanolic Extracts on Oxidative Status

The concentrations of oxidative indices (SOD, GPx, MDA) are shown in Table 4.3. The results revealed the concentration of SOD to be 30.36 ± 1.36 and 27.38 ± 0.60 in normal control and the induced group respectively. Administration of 200mg/kg seed extract, 200mg/kg leaf methanolic extract, 400mg/kg seed/leaf extracts and 400IU vitamin E (VE-standard) revealed the concentration of superoxide dismutase to be 28.71 ±0.23 , 28.55 ± 0.45, 28.98 ± 0.90, and 29.77 ± 0.53 (µg/mg) in the test groups (3, 4, 5 and 6) respectively.

The concentration of GPx were 23.89 ± 0.71 and 21.18 ± 0.43 in groups 1 and 2 respectively. The administration of the extracts and the standard drugs showed the concentrations of 22.23 ± 0.13, 22.20 ± 0.34, 22.18 ± 0.43, 22.84 ± 0.17 (µg/mg) in group 3,4 5 and 6 respectively.

Also, the results of MDA revealed the concentration of 5.25 ± 0.25 and 12.78 ± 0.65 in group 1 and 2 respectively. The treatment with the extracts 200mg/kg seed extract, 200mg/kg leaf extract, 400mg/kg seed/leaf extracts and 400IU vitamin E (VE-standard) showed the concentration to be 8.25 ± 0.25, 9.21 ± 0.45, 10.23 ± 0.25, and 9.22 ± 0.45 in the group 3, 4, 5 and 6 respectively.

Table 3: Effect of Leaf and Seed Methanolic Extracts on the Oxidative Status

Groups	SOD(µg/mg)	Gpx (µg/mg)	MDA (nmol/ml)
1	30.36 ± 1.36[b]	23.89 ± 0.71[b]	5.25 ± 0.25[a]
2	27.38 ± 0.60[a]	21.18 ± 0.43[a]	12.78 ± 0.65[d]
3	28.71 ± 0.23[b]	22.23 ± 0.13[b]	8.25 ± 0.25[b]
4	28.55 ± 0.45[b]	22.20 ± 0.34[b]	9.21 ± 0.45[c]
5	28.98 ± 0.90[b]	22.18 ± 0.43[b]	10.23 ± 0.25[c]
6	29.77 ± 0.53[b]	22.84 ± 0.17[b]	9.22 ± 0.45[c]

The results are expressed in Means ± SD (n = 5). Mean values with different letters as superscripts going down the group are considered significant at $P< 0.05$

Group 1 = Normal control, Group 2 = Induced (20mg/kg $AlCl_3$) untreated, Group 3 = 200mg/kg seed extract

Group 4 = 200mg/kg leaf extract, Group 5 =400mg/kg seed/leaf extracts, Group 6 = 400IU vitamin E (standard)

Effect of Leaf and Seed Methanolic Extracts *of C. Pepo* L on IL-6

The results of the analysis of the levels of interleukin-6 (IL-6) are shown in table 4.4. The concentration IL-6 was showed to be 195.17 ± 8.63 and 226.89 ± 3.45 **(**pg/mL**)** in group 1 and 2 respectively. The administration of the extracts and standard drugs showed the concentrations to be 215.91 ± 20.71, 207.37 ± 13.80, 200.66 ± 11.21, 207.98 ± 4.31 (pg/mL) in the test groups respectively

Table 4: Effect of the Leaf and Seed Methanolic Extracts on IL-6

Groups	IL–6 (pg/mL)
1	195.17 ± 8.63[a]
2	226.89 ± 3.45[e]

Groups	IL–6 (pg/mL)
3	215.91 ± 20.71[d]
4	207.37 ± 13.80[c]
5	200.66 ± 11.21[b]
6	207.98 ± 4.31[c]

The Results are Expressed as Means ± SD (n = 5) Mean Values with Different Letters as Superscripts Down the Group are Considered Significantly Different at $p < 0.05$

Group 1 = Normal control, Group 2 = Induced (20mg/kg $AlCl_3$), Group 3 = 200mg/kg seed extract,
Group 4 = 200mg/kg leaf extract, Group 5 = 400mg/kg seed/leaf extracts, Group 6 = 400IU vitamin E (standard)
IL-6 = Interleukin 6.

Effect of Leaf and Seed Methanolic Extracts on Reproductive Hormones

Table 4.5 shows the varying effect of the sample extracts on reproductive hormonal levels. Results of follicle stimulating hormone (FSH) and luteinizing hormone (LH) revealed no significant (P>0.05) difference between the normal control group and all the treatment groups. In contrast, progesterone concentration was revealed to be 27.04 ± 0.71(ng/mL) and 31.00 ± 0.63(ng/mL) in the group 1 and 2 group respectively. The administration of the extracts showed the concentration to be 29.22 ± 0.16, 28.32 ± 0.79, 28.55 ± 0.63 and 30.00 ± 0.32(ng/mL) following postpartum hormonal alteration in the groups treated with 3,4, 5 and 6 respectively.

Table 5: Effect of Leaf and Seed Methanolic Extracts on Reproductive Hormones

Groups	FSH (ng/mL)	LH (ng/mL)	Progesterone (ng/mL)
1	0.56 ± 0.01[a]	1.35 ± 0.11a	27.04 ± 0.71[a]
2	0.61 ± 0.03[b]	1.55 ± 0.02[b]	31.00 ± 0.63[d]
3	0.59 ± 0.3[a]	1.37 ± 0.06[a]	28.55 ± 0.63[b]
4	0.59 ± 0.03[a]	1.50 ± 0.43[b]	28.32 ± 0.79[b]
5	0.58 ± 0.02[a]	1.38 ± 0.02[a]	29.22 ± 0.16[c]
6	0.56 ± 0.02[a]	1.44 ± 0.07[a]	30.00 ± 0.32[c]

The Results are Expressed as Means ± SD (n = 5). Mean Values with Different Letters as Superscripts Down the Group are Considered Significantly Different at $p < 0.05$. Group 1 = Normal Control, Group 2 = Induced (20mg/kg $AlCl_3$), Group 3 = 200mg/kg Seed Extract. Group 4 = 200mg/kg Leaf Extract (L), Group 5 = 400mg/kg Seed/Leaf Extracts (S/L), Group 6 = 400IU Vitamin E (Standard), FSH = Follicle Stimulating Hormone, LH = Luteinizing Hormone, P4 = Progesterone

Table 6: Result of the Pups Produced in Response to the Extracts Administered (Pregnancy Outcomes)

Groups	Number of Pregnancies	Number of Pups
1	Once	25
2	Nil	Nil
3	Once	28
4	Once	30
5	Once	38
6	Once	38

Group 1 = Normal Control, Group 2 = Induced (20mg/kg $AlCl_3$), Group 3 = 200mg/kg Seed Extract, Group 4 = 200mg/kg Leaf Extract (L), Group 5 = 400mg/kg Seed/Leaf Extracts (S/L), Group 6 = 400IU Vitamin E (Standard)

Discussion

In the present study, the preliminary qualitative analysis of pumpkin leaf methanolic extract revealed the present of alkaloids, saponins, phenols, tannins, steroids, flavonoids cardiac glycosides and glycosides while terpenoids was shown to be absent. The study also revealed significant amounts of flavonoids (23.62 ± 0.57 mg/g), phenols (17.99 ± 1.47 mg/g), alkaloids (37.15 ± 0.30 mg/g) and terpenoids (25.79 ± 0.62 mg/g) in the quantitative compositions of pumpkin seeds. However, alkaloids, terpenoids, and flavonoids were revealed by Butte *et al*. (2019) to have anti-inflammatory activity. Similarly, the study revealed for its various pharmacological action such as hepatoprotection, inhibition of benign prostatic hyperplasia, antioxidant, anticancer, antimicrobial, anti-inflammatory, antidiabetic, and antiulcer (Jahan *et al*., 2023).

Antioxidant activity involves the use of compounds or mixtures to reduce pro-oxidants, free radicals, or reactive species (Olszowy and Dawidowicz, 2018). Researchers have discovered that polyphenols and flavonoids help plants deal with a wide range of stressors. There are numerous phenolic chemicals in plants that are used in important ways (Kumar *et al*., 2020).

According *to Kangralkar et al.,* (2010) Superoxide dismutase (SODs) are described as a group of metalloenzymes and a key cellular antioxidant, responsible for the elimination of oxygen radical (O_2^-). These are found in all kingdoms of life. SODs form the front line of defense against reactive oxygen species (ROS)-mediated injury *(Kangralkar et al.,* 2010). The prime interest in the therapeutic treatment of oxidative stress is SODs because of their smaller size, longer half-life, and similarity in function to the native enzyme *(Salvemini et al., 2000).* The induction of female infertility using $AlCl_3$ significantly ($p<0.05$) decrease the serum concentration of SOD in the induced group. However, treatment with the extracts (200mg/kg seed, 200mg/kg leaf 400mg/kg seed & leaf in 1:1 mixtures and 400IU vitamin E) ameliorated the oxidative stress induced by significantly ($p<0.05$) increasing the serum concentration of SOD across all the groups when compared to the induced group. This is due to the potential mechanism of $AlCl_3$ disruptions effect on ovary structure and functions, and metabolic imbalanced of trace minerals. Which decreased the contents of follicle stimulating hormone and luteinizing hormone revealed by (Wang *et al.*, 2011). Furthermore, $AlCl_3$ altered both spermatogenesis and steroidogenesis through depletion in the secretion of Human Gonadotropin Releasing Hormone (GnRH) (closes to calcium channel on the hypothalamus) thereby affecting FSH and LH. $AlCl_3$ may also affect the calcium channel in sertoli and leydig cells thus impairing the synthesis of androgen (Geeta *et al*., 2017). The results further showed that there was no significant ($p > 0.05$) increase between the groups treated with 200mg/kg seed, 200mg/kg leaf and 400mg/kg Seed & Leaf) but the serum concentration of SOD increase significantly ($p<0.05$) in group 6 (vitamin E) when compared to group 3, 4 & 5, which might be due to slight change in the doses of the standard drugs. This agree to the study carried out by Sarah *et al.*, (2022) that the pumpkin seeds scavenged free radical in the concentration dependent manners.

Glutathione peroxidase (GPx) aids reproduction and pregnancy through detoxification of peroxides (hydrogen peroxides and lipid peroxides) forming harmless products and thereby preventing damage to cell by reactive oxygen species (Kryukov., *et al* 2003). The present study showed significant ($p<0.05$) increase in the serum concentration of GPx across all the groups compared to the induced group. However, there was no significant difference ($p>0.05$) between the treated groups. According Mehdi *et al*., (2013), GPx and other trace elements like Zn are strongly associated with follicular fluid and fertility disorders

Malondialdehyde (MDA) is a product of lipid peroxidation and is the most frequently and reproducible evaluation in assessing bimolecular damage (Marrocco *et al*., 2017).

In this study, the serum concentration of MDA was significantly higher in group 2 (induced) when compared to group 1 and the treatments groups. This showed that $AlCl_3$ caused mild lipid peroxidation in the rats. Thus, the induction of infertility by $AlCl_3$ significantly decrease ($p<0.05$) the serum concentration of SOD and GPx and significantly ($p<0.05$) elevated the serum concentration of MDA across all the groups when compared to group 1 and group 2 with concomitant increased in the serum concentration of SOD and GPx and decreased in the MDA serum concentration following the administration of the extracts. This could be as results of antioxidants present in the extracts which agrees with the report of Brogan *et al*. (2016) that pumpkin plant has antioxidant and anti-inflammatory activity. This is similar to the findings of Dang (2004) which revealed that pumpkin extracts supplementation significantly raised the serum and hepatic activities of glutathione peroxidase and superoxide dismutase in mice and decreased the quantity of malondialdehyde. This showed that the extracts have antioxidant properties and therefore, have propensity of attenuating oxidative stress.

Previous study by Mukherjeer *et al*. (2014) have discovered that elevated levels of intracellular glutathione result in a healthier and stronger embryo. Indeed, all the treated groups after inducing yielded reproductive outcome.

Malvezzi *et al*. (2019) described Interleukin-6 (IL-6) as the most studied interleukin in endometriosis which has important functions in reproductive physiology. Alteration of this cytokines concentrations may have linked to fertility anomaly (Li *et al*., 2021).

From this finding, the result of interleukins 6 cytokines showed significant ($p<0.05$) increase in all the groups when compared to group 1 with 195.17 ± 8.63 **(**pg/mL**)** and decrease significantly after the administration of the extracts across all the groups when compared to group 2 with 226.89 ± 3.45 (pg/mL**).** The result also showed varying effect of the extracts on the serum concentration of the cytokines as the group treated with 400mg/kg Seed & Leaf significantly decrease the serum concentration of IL-6 to 200.66 ± 11.21 when compared the to the group treated with the 200m/kg Leaf extract with concentration of 207.37 ± 13.80 and the standard group with concentration of 207.98 ± 4.31. On the other hand, the group treated with just 200mg/kg of Seed extract revealed the serum concentration of 215.91 ± 20.71. The significantly ($p>0.05$) higher concentration of serum IL-6 in the group 2 could be an indication of oxidative stress induced and inflammation or uterine disorders. Equally, the significant ($p<0.05$) decrease in the serum concentration of IL-6 in the test group might be as results of the doses as it was revealed by Sarah *et al.,* (2022) that the pumpkin seeds scavenged free radical in the concentration dependent manners. Although it has potency since all the treated exhibited some level of improvements. As all the treatments groups expressed outstanding respond by given birth.

Figure 2: *Cucurbita pepo*

However, the group 3 treated with 400mg/kg of both *Cucurbita pepo* leaf and seed showed low level of IL-6 serum concentration compared to group 2 and all the treatment group, nevertheless is still higher than the normal control group. This also expressed that, the combination of both leaf and seed of the plant extracts would have great physiological function on inflammatory cytokines and could be more effective than the standard drugs (vitamin E).

Hormones is a class of signaling molecules in organisms (animals, plants and fungi) development that are sent to distant organs or tissues by complex biological processes to regulate physiology and behaviors *(Shuster, 2014).*

Follicle-stimulating hormone (FSH) is a gonadotropin glycoprotein polypeptide hormone which are synthesized and secreted by the gonadotropic cells of the anterior pituitary gland and regulates the development growth, pubertal maturation and reproductive processes. Both follicle stimulating hormone and luteinizing hormones work synergistically (*Cahoreau et al*., 2015). *Bowen, 2019 described Luteinizing hormone (lutrophin) as a hormone produced by gonatrophic cells in the anterior pituitary gland which is regulated by gonadotropin releasing hormone (GnRh) from the hypothalamus (GCSE, 2022). In females, an acute rise of LH trigger ovulation (Stamatiades et al., 2018) and development of corpus luteum. In males, where LH had also been called interstitial cell stimulating hormone (ICSH) (which stimulates lydig cell production of testosterone. LH work synergistically with FSH (Nosek, 2016).* Progesterone (P4) is an endogenous steroid which involved in the menstrual cycle, pregnancy, embryogenesis of human and other species *(Jameson, 2015*). This belongs to the class of major steroid hormone in the body called progestogens (King*, 2010)*.

In this study, the result of follicle stimulating hormone (FSH) and luteinizing hormone (LH) *showed no significant (p>0.05) difference across the treatment groups except in the group 2 which was not treated and hence there was no conception observed despite the concentration of FSH, LH and progesterone.* There was significant (p<0.05) difference in group 2 and 6 when compared Group 1, 3 and 5 with the lowest concentration. This agrees to the findings of Lamotte *et al*. (2004) that, the level female reproductive hormones (FSH, LH and P4) decrease dramatically following childbirth to stimulates breast milk secretions through the process of lactogenesis which is the normal physiological processes. Interestingly, despite the hormonal concentration in group 2, there was no conception which could be attributed to the oxidative stress as indicated by the high serum concentration of MDA and IL-6 in group 2.

Limitations of the Study

There was a sudden change of experimental animals from rabbits to albino rats.

Seasonal scarcity of experimental animals causes high demand and cost inflation.

This work was limited to laboratory animal studies

CONCLUSION

This research determined the therapeutic effects of *Cucurbita pepo* leaf and seed extracts against infertility induced by oxidative stress. From the findings of the present study, pumpkin plants are rich in antioxidants that ultimately have modulatory effects on inflammation and oxidative stress. The group treated with 200mg/kg of leaf and seed extracts (combined) showed remarkable improvement over the group treated with just seed or leaf of 200mg/kg. Though, all the treated groups showed protective effects compared to untreated group. This is an indication that $AlCl_3$ induced oxidative stress and chronic oxidative stress can affect the quality of oocytes, destroy uterine lining and degrades follicular fluids, induced persistence luteal or reduced clearance. All these increases the risk of infertility. Following the significant increase in the serum level of primary antioxidant activity (SOD and GPx) and significant decrease of serum level concentration of MDA and inflammatory cytokines (IL-6) in all the treated groups compared to the induced group, It is and thus confirmed the protective effect of extracts administered and showed that fertility disorders due to oxidative stress and inflammation could be a life time's problems.

Recommendations

i. Histology examination of uterus and ovaries should further be analyzed to establish the extent of damage caused by $AlCl_3$ induced.
ii. Similar study should be carried out on male infertility to diagnose the effect of the extracts on sperm physiology and pathology.
iii. Pumpkin plants should be included as normal diet routine even without manifestation of any disorder to boost immunity. Since one of the known side effect is the upset of the stomach, it can be transformed into different dish like cake, bread and sauce.
iv. Infertility management and treatment should be carried along with inflammatory markers investigation and oxidative stress mitigation for effectiveness. since hormonal disorder alone may not absolutely account for the problem of female infertility.

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