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Protective effect of mixture of honey and *Garcinia* kola extract against cyclophosphamide–induced reproductive toxicity in male albino rats

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Abstract

Cyclophosphamide is a widely used drug for the treatment of many human malignant tumors. This study evaluated the protective potentials of fresh honey and *Garcinia* kola extract against reproductive toxicity caused by Cyclophosphamide in male Wistar rat. Group A served as the negative control and were administered feed and normal saline (2 ml/kg bw) daily for six weeks by oral gavage. Group B served as positive control and received feed and Cyclophosphamide (100mg/kg bw) via injection after 24hrs was given normal saline for six weeks. Groups C, D and E received Cyclophosphamide (100 mg/kg bw) via injection after 24hrs was respectively administered fresh honey (2ml/kg bw), a mixture of *Garcinia* kola and unprocessed Honey (2 ml/kg bw) plus *Garcinia* kola extract (100mg/kg bw) for the next six weeks. Animals in groups C, D and E revealed increased in body weight gain and this was statistically significant at $p<0.05$. The final body weight of the cyclophosphamide treated rats was significantly reduced from 183.2±8.02 to 195.10±8.08. A significant ($P<0.05$) reduction in the weight of the testis of the rats administered with cyclophosphamide compared with the positive control was observed (0.93±0.050 vs 1.22±0.15). However, post treatment with fresh honey, *Garcinia* kola and a combination of honey and *Garcinia* kola significantly improved the weights of testes compared with positive control (Group C: 1.13±0.05 vs 0.93±0.05; Group D: 1.20 ±0.020 Vs 93±0.05; Group E: 1.18±0.040 vs 93±0.05). The serum concentration of Luteinizing Hormone, Follicle Stimulating Hormone and testosterone was significant ($P<0.05$) reduced in group B rats compared with Group A rats. Separate treatments with *Garcinia* kola extracts and unprocessed honey cause an elevation in serum LH, FSH and testosterone levels compared with the positive control rats. The mixture of *Garcinia* kola extracts and fresh honey improved serum levels LH, FSH and testosterone significantly ($p<0.05$) (Group E) compared with group B. The results obtained indicated that fresh honey and *Garcinia* kola either used separately or in combination can ameliorate Cyclophosphamide (CPA) induced reproductive toxicity in rats.

Keywords: *Garcinia* Kola; Honey; Cyclophosphamide; Reproductive Toxicity; Reproductive Hormone

1. Introduction

The endocrine system controls the body's metabolism and produces several hormones responsible for reproductive activities in humans and animals. The reproductive function is specifically regulated by hormones produced/secreted by the hypothalamus, pituitary gland and the gonads [1]. These vital organs play a key role in hormonal regulation by

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allowing cyclic production of gonadotropic and steroid hormones [2]. The male gonads (testes) could be exposed to chemicals that have negative effect on its development and function leading to infertility or subfertility. Male infertility is caused by several factors and could be attributed to intra- and/or extra-testicular factors [3]. Majority of these factors are known to induce alterations in sperm parameters such as low sperm concentrations, poor sperm motility, abnormal sperm morphology, and impairment in DNA and chromatin integrity [4]. Factors reported to cause fertility disorders includes; drugs, chemotherapy, radiotherapy, apoptosis, radical oxidative species (ROS), and smoking [5].

Cyclophosphamide (CP) is an oxazophosphorine class of alkylating agent that is frequently and widely used drugs for the treatment of several human malignant tumors [6-8]. It is a cytotoxic alkylating agent with anti-tumor and immunosuppressant properties used for the management of breast cancer, non-Hodgkin's lymphoma, acute myeloid leukemia, chronic myelogenous leukemia, solid tumours and acutelymphoblastic leukemia [9-10]. However, despite its therapeutic significance and benefits, its medical application has been limited to its adverse effects on the body system, including reproductive toxicity [6-7].

Several studies have confirmed that increased exposure to metabolites of cyclophosphamide can induce reproductive toxicity in humans and animals [11]. The adverse effect of CP on reproductive potential includes; low sperm count and motility [12-14], non-functionality of normal spermatogenic cycle in testis, permanent infertility at higher doses [15], and loss in the weight of reproductive organs (testes) leading to infertility [16]. The two active metabolites of CP are Phosphoramide mustard and acrolein [17]. The antineoplastic effect of CP is related with phosphoramide mustard, while acrolein is connected to toxic effects such as cell death, apoptosis, oncosis, and necrosis [18]. A study by Rezvanfar *et al*., [19] indicated that CP alters human reproductive system causing infertility. Similar studies on laboratory animals have established the ability of CP to cause testicular weight loss, transitory oligospermia, reduced DNA and impaired spermatogenesis and androgenesis and induced germ cells apoptosis, and histological alterations in the testes and epididymis [19-20]. The exact mechanism of CP induced-testicular toxicity is unclear, though; it may be connected to the action of the highly reactive metabolite acrolein via the generation of reactive oxygen species (ROS), resulting in oxidative stress [21], and also its interference with tissue antioxidant defense system, thus producing highly reactive oxygen free-radicals that are mutagenic to mammalian cells [22].

Medicinal plants have been demonstrated to have beneficial properties for the treatment and prevention of several diseases in humans. They possess active phytochemical ingredients responsible for their pharmacological activities and secondary metabolites that can protect humans against diseases [23-24]. In recent times, several authors across the globe have become interested in potential compounds of plant origin that are capable of alleviating the adverse effects of chemotherapy on normal cells without compromising its anti-neoplastic activity [25]. One of such plants is *Garcinia* kola, a plant which belongs to the family *Clusiaceae guttiferae*. It contains a complex mixture of biflavonoids, prenylatedbenzophenones and xanthones [26]. *Garcinia* kola seed is a highly valued ingredient in African ethno medicine [27], which is used as an antidote for ingested poison, and as a cure for abdominal colicky pain, chest cold, cough and hepatitis [28]. Scientifically, the potentials of Garcinia kola as a therapeutic agents has been documented and it includes; anti-inflammatory, hepato-protective, purgative, antimicrobial, antithrombotic and antiviral properties [29]. The seeds of Garcinia kola contain proteins, lipids, carbohydrates and minute quantities of Kolaviron (biflavonoids GB-1, GB-2 and Kolaflavone) [30]. Many researchers have reported that *Garcinia* kola seed bioactivity is connected to the presence of bioflavonoids, which are well known antioxidants [31]. In Nigeria, the seeds of *Garcinia* kola are used in many herbal preparations for the treatment of diseases such as liver disease, laryngitis, bronchitis and diarrhea [30].

Honey is a thick, viscous and sweet liquid substance produced when the nectar of flowers are gathered, transformed and stored in the honeycomb by honey bees [32-33]. The nectar consists of water (80%), fructose (38.5%) and glucose (31.0%), maltose, sucrose, proteins, phenolic compounds, vitamins and minerals [34-35]. Scientific studies carried out in laboratory animals have convincingly that Honey possess several health benefits such as gastroprotective, hepatoprotective, nephroprotective [36], hypoglycemic, antioxidant [37], antihypertensive, antibacterial, antifungal, and anti-inflammatory effects [38-39]. Honey also have anti-tumor and anti-metastatic properties and potentiates the anti-tumor effects of cytotoxic drugs [38]. Mohammed and colleagues [40] reported that honey at a dose 1.2 g kg/day for 13 weeks, increases the number of Leydig cells, seminiferous tubules diameter, and epithelial heights of the testes in male rats with cigarette smoke-induced testicular damage.

Several experimental studies have demonstrated that the protective potential of some medicinal plant extracts are associated with abundant phenolic compounds found in such plants. These medicinal plants serve as good storage of pharmaceutical and chemical templates for new drugs formulation. It is on this basis that this study was carried out to investigate the protective potentials of the mixture of *Garcinia* kola extract and fresh honey against cyclophosphamide-induced reproductive toxicity of male rats.

2. Material and methods

2.1. Chemicals

All chemicals used were of analytical grade, Ethanol, 30mM Hydrogen peroxide, 6M Hydrogen tetraoxosulphate(H_2SO_4), Tris Amino methane buffer (hydroxyl methyl), Phosphate buffer, Carbonate buffer, $KMNO_4$, NaCl, NaOH,Na_2HPO, HCl,$Na_2CO_3NaHCO_3$, EDTA (Ethylene diamide tetra acetic acid), Anrenaline solution.

2.2. Preparation of *Garcinia* Kola Extract

Garcinia kola seeds were purchased from the local Swali market in Yenagoa, Bayelsa State, Nigeria. The *Garcinia* Kola extract was prepared according to the method Iwu et al. [41]. Peeled seeds were sliced and air dried and then pulverized in a blender. Powdered seeds were extracted with n-hexane in a soxhlet tube for 24hours. The defatted dried mare was repacked and extracted with methanol. The extract was concentrated with chloroform. The concentrated extract called kolaviron is a golden yellow solid [41].

2.3. Honey Collection

Fresh honey obtained from the Forest of Wilberforce Island, Amassoma, Bayelsa State was bought from the honey farmer by the researcher. It was taken to the department of Biological Sciences and was identified and confirmed by Dr. Ayodeji A.

2.4. Animals

Twenty-five (25) healthy male rats weighing 120-180g were purchased from the Department of Pharmacology Animal House, Niger Delta University, Bayelsa State. The rats were housed in cages made of metal nesting under the standard environmental conditions of a 12hr light/dark cycle and were fed with growers mash and distilled water *ad libitum*. The beddings were changed and the cages cleaned every morning and disinfected at interval of three days. The rats were allowed to acclimatize for 14 days before experimentation. The total experimental period was 8 weeks. The experimental protocol was presented to the Ethical Committee of College of Health Sciences, Niger Delta University Wilberforce Island for approval before the commencement of the work. The Animal Welfare Act of 1985 of the United States of America for research and Institutional Animal Care and Use Committee (IACUC) protocol was strictly followed.

2.5. Experimental Design

Twenty-five healthy male albino rats were used for the experiment. The male albino rats, after acclimatization period were randomly divided into five (5) groups A, B, C, D and E; comprising five (5) rats in each group.

- Group A: Normal Control: served as the negative control and were administered feed and normal saline (2ml/kg bw) daily for six weeks by oral gavage.
- Group B: Positive Control: positive control and received feed and Cyclophosphamide (100mg/kg bw) via injection after 24 hrs was given normal saline (2ml/kg bw) daily for six weeks
- Group C: received Cyclophosphamide (100mg/kg bw) via injection after 24hrs was administered fresh honey (2ml/kg bw) daily for the next six weeks.
- Group D: received Cyclophosphamide (100mg/kg bw) via injection after 24hrs was administered (100mg/kg bw) of Garcinia kola extract daily for the next six weeks.
- Group E: Received Cyclophosphamide (100mg/kg bw) via injection after 24hrs was administered a mixture of Garcinia kola and unprocessed Honey (1:2 w:v) (2ml/kg bw) daily for the next six weeks

The cyclophosphamide was administered via injection after 24hrs. *Garcinia* kola extracts and unprocessed honey was administered once daily by oral gavage for six (6) weeks. At the completion of the treatment; the rats were anaesthetized by inhalation with diethyl ether and sacrificed. Blood samples were collected via cardiac puncture into plain sample glass containers. The blood were allowed to cloth properly at room temperature and centrifuged at 3,000rpm for 5miutes to obtain serum. The clear serum was collected in sterilized disposable plastic tubes and stored at 2 – 8^{o}C until analysis.

2.6. Estimation of Reproductive Hormones

Serum levels of Follicle stimulating Hormone, Luteinizing Hormone, and testosterone were determined using specific commercial kits (IBL-Hambug GmbH, Germany). These hormones were measured in serum by standard operational procedure using specific commercial kits on the principle of Enzyme Linked Immunosorbent Assay (ELISA) method as

described by Steyn et al. [42]. The assays were performed in accordance with the manufacturer's protocols as stated in the manuals. Using the correct wavelength for each analytes, the absorbance was read with a microtitre plat reader, after which the corresponding concentration was calculated.

2.7. Statistical Analysis

All data obtained were presented as mean and standard deviation (Mean ±SD). The SPSS Software of version 23.0 was used for the analysis of the data obtained. Comparison of result between control and test was done using one-way analysis of variance (ANOVA and group means were compared using Bonferroni multiple comparison. Level of significance was determined at a probability level of $p< 0.05$.

3. Results

Table 3.2 shows the comparison of Mean Body Weight of male Albino Rats before the Experiment and prior to sacrifice in the control and Experimental Group. The result revealed that animals in the groups administered *Garcinia* kola, Honey and a mixture of both Honey and *Garcinia* kola (ie C, D and E) showed increased in body weight gain and this was statistically significant at $p<0.05$. Also, result revealed a statistically significant ($P<0.05$) decrease in the final body weight of the cyclophosphamide treated rats compared to their initial body weight (183.2±8.02Vs195.10±8.08). Table 3.2 shows the toxicological study of oral administration of Cyclophosphamide, and therapeutic study of Honey and *Garcinia* kola Extract on weight of testis of adult male Albino Rats. The study revealed a significant ($P<0.05$) reduction in the weight of the testis of the rats administered with cyclophosphamide (group B) (0.93±0.050) compared with control (Group A) (1.22±0.15). However, following treatment with fresh honey and a mixture of honey plus *Garcinia* kola extract to group C and E rats respectively demonstrated a slight increase in the weight of the testis compared with group B rats(Group C: 1.13±0.05Vs 0.93±0.05; Group E: 1.18 ±0.02 Vs 0.93±0.05). In Group D (1.20 ±0.02) rats that were administered with *Garcinia* kola extracts alone showed that the testicular mean weight was restored to that of negative control group A (1.22±0.15) indicating a testicular protective effects (move to discussion). Table 3.3 showed the effect of the cyclophosphamide and a mixture of *Garcinia* kola extracts and fresh honey on some male reproductive hormones of Cyclophosphamide induced-Reproductive toxicity in Male Albino Rats. The result showed a statistically significant ($P<0.05$) reduction in the serum concentration of LH, FSH and testosterone in the cyclophosphamide intoxicated rats (group B) compared with the control (Group A). Post treatment with *Garcinia* kola extracts and unprocessed honey separately caused an elevation in serum LH, FSH and testosterone levels as compared with the cyclophosphamide intoxicated rats. However, the administration of a mixture of *Garcinia* kola extracts and fresh honey on the cyclophosphamide intoxicated rats of group E resulted a significant ($p<0.05$) elevation in the serum LH, FSH and testosterone levels when compared with the cyclophosphamide only treated rats (Group B).

Table 1 Comparison of Mean Body Weight of Male Albino Rats in the Control and Experimental Groups before the Experiment and prior to sacrifice

Group	Initial body weight X±SD	Final body weight X±SD	% Body Weight Gain	P-value
Group A	170.10± 8.15	199.6±10.03	9.5	.000*
Group B	195.10±8.08	183.2±8.02	12.1	.010*
Group C	166.09±9.10	190.9±8.11[c]	24.81	.000*
Group D	160.20±7.74	188.05±8.27	27.85	.000*
Group E	169.05±6.24	197.3±9.97	28.25	.000*

Key: SD= standard deviation; bc= statistically Significant; a=not significant; Group A= Control (Feed and water); Group B = Cyclophosphamide only (100mg/kg body weight). Group C= Cyclophosphamide (100mg/kg body weight) + and Honey (2ml/kg body weight) administered group. Group D= Cyclophosphamide (100mg/kg body weight) + and Garcinia kola extract (100mg/kg body weight) administered group. Group E= Cyclophosphamide (100mg/kg body weight) + mixture of Garcinia kola and honey (2ml/kg body weight) administered group.

Table 2 Toxicological Study of Oral Administration of Cyclophosphamide, and therapeutic study of Honey and *Garcinia* kola Extracts on Organs Weight (Testis) of Adult Male Albino Rats

Group	Group A (Normal Saline)	Group B (CPA only)	Group C (CPA+Honey)	Group D (CPA + *Garnicia* kola)	Group E (CPA + *Garnicia* kola + Honey)
Organ (Testis)	1.22±0.15[a]	0.93±0.05[b]	1.13±0.05[c]	1.20±0.02[c]	1.18±0.04[d]

Values expressed as Mean ±SD; Values with the same superscripts are not significant (P>0.05)

Table 3 Effect of the mixture of Honey and *Garcinia* kola on Reproductive Hormones of Cyclophosphamide induced-Reproductive toxicity in Male Albino Rats

Group	LH (mμ/L) X±SD	FSH (mμ/L) X±SD	Testosterone (mg/ml)X±SD
Group A	19.76±3.21[a]	29.33±3.93[a]	151.60±7.60[a]
Group B	9.90±2.60[b]	11.79±4.17[b]	124.65±9.60[b]
Group C	18.63±1.77[a]	28.80±3.92[a]	150.70±9.60[a]
Group D	17.53±1.77[c]	28.00±2.82[a]	148.67±10.40[a]
Group E	22.90±1.43[c]	32.30±3.30[c]	154.10±6.70[c]

Key: SD= standard deviation; bc= statistically Significant; a=not significant; Group A= Control (Feed and water); Group B = Cyclophosphamide only (100mg/kg body weight).Group C= Cyclophosphamide (100mg/kg body weight) + and Honey (1ml/kg body weight) administered group. Group D= Cyclophosphamide (100mg/kg body weight) + and Garcinia kola extract (100mg/kg body weight) administered group. Group E= Cyclophosphamide (100mg/kg body weight) + mixture of Garcinia kola and honey (2ml/kg body weight) administered group.

4. Discussion

Cyclophosphamide (CP) is an anti-cancer chemotherapeutic agent used for the treatment of multiple malignancies and autoimmune diseases [43-44]. Several authors have documented that cyclophosphamide causes injuries to many organs in mammals including; liver, lung, spleen, kidneys, heart and testes [45-46]. The exact mechanism of cyclophosphamide deleterious effect on body cells/tissues could be attributed to its metabolites: phosphoramide mustard and acrolein, through the generation of toxic reactive oxygen species (ROS) [47], which interacts with protein, amino acids causing structural and functional changes [48]. Medicinal plants have been shown to have beneficial properties for the management of several diseases. They possess active phytochemical ingredients that have antioxidant properties responsible for their pharmacological activities; and secondary metabolites that can protect humans against diseases [22-24]. The current study investigated the protective effect of mixture of fresh honey and *Garcinia* kola extract on cyclophosphamide induced reproductive toxicity in male albino rats.

In the present study, cyclophosphamide caused a statistically significant ($p<0.05$) decrease in the mean body weight of all the experimental rats compared with the control as shown in (table 1). The final mean body weight of cyclophosphamide treated rats (group B) was lower than initial body weight with a percentage weight loss of 12.10%. This is consistent with reports previous studies by Khorwal et al. [48]; Kumar et al., [49]; Myers et al., [50] and Emmenegger et al. [51], which reported significant weight loss in laboratory animals treated with cyclophosphamide. This loss in weight could be attributed to the degenerative changes seen in the several body organs leading to loss of appetite and decrease in food intake, or due to a direct effect upon energy metabolism, and anti-proliferative effects of cyclophosphamide on adipocyte progenitors [50]. However, the rats in groups C and D post treated with fresh honey and *Garnicia* kola extract respectively showed an elevated final mean body weight than the initial body weight with a percentage weight gain of 24.81% and 27.85% respectively. Furthermore, in rats of group E post treated with a mixture of honey and *Garnicia* kola extract showed a much higher final weight gain than the initial body weight with a percentage weight gain of 28.25%. This confirmed that co-administration of honey and *Garnicia* kola extract is associated with significant body weight gain. Honey and *Garnicia* kola effect on body weight could be attributed to its antioxidant effect, and also, its capacity to improve general well-being resulting in increased food intake, feed efficiency hence, preventing weight loss or causing weight gain [52].

Furthermore**,** the study revealed a statistically significant ($P<0.05$) reduction in the weight of the testis of the rats administered with cyclophosphamide compared with control as shown in (table 2). This result is similar to the findings of Hutheyfa and colleagues [53], who reported a reduced testicular weight of cyclophosphamide administered rats.

However, following treatment with fresh honey, *Garnicia* kola extracts and a mixture of fresh honey and *Garnicia* kola extracts on the cyclophosphamide intoxicated rats (Group C, D and E respectively), the result showed that the testicular mean weight was similar to the control group. This suggests that fresh honey, *Garnicia* kola extracts and a mixture of fresh honey and *Garnicia* kola extracts possess testicular protective effects.

CP is known to be toxic to the male reproductive system, especially the testes and epididymis. Cyclophosphamide was shown to disturb the male reproductive hormones at the gonadal levels. Several studies on cyclophosphamide-induced gonadal toxicity in human and rodents indicated an association with oligospermia, azoospermia, reduced testicular weight, alterations in gonadotrophin, alterations in testosterone levels/oxidative stress parameters and testicular tissue toxicity [52,54,55]. Normally, the testes synthesize two important products: testosterone and sperm needed for male fertility. The synthesis of both products is regulated by endocrine hormones produced in the hypothalamus-pituitary axis and the testis. The release of hypothalamic gonadotropin-releasing hormone (GnRH) triggers production of LH and FSH by the pituitary gland. The LH is travels through the circulation to the testes, where it stimulates Leydig cells to produce testosterone. In return, the testes give feedback on the hypothalamus and the pituitary through testosterone and inhibin secretion, in a negative feedback loop to limit GnRH and gonodotropin production [56]. Disruption of the hypothalamic-pituitary axis and testis by certain compounds such as cyclophosphamide can impact negatively on the regulatory function of the GnRH and influence the level of male reproductive hormones. In the present study as shown in (table 3), cyclophosphamide treatment was associated with decreased serum LH, FSH and testosterone levels in the cyclophosphamide intoxicated rats (group B) compared with the control (Group A). This confirms the report of Onaolapo and colleagues [52], which reported a significant reduction in LH, FSH and testosterone levels. Post treatment of the cyclophosphamide intoxicated rats with the fresh honey and *Garcinia* kola extract alone/separately and in combination (Group, C, D and E respectively) significantly ($p<0.05$) improved the concentrations of these hormones when compared with the cyclophosphamide intoxicated group B as shown in (table 3). This suggests that *Garcinia* kola extracts and fresh honey administration is associated with mitigation of cyclophosphamide-induced gonadal toxicity in rats as manifested by the elevated level of the hormones in this study. However, the group treated with the combination of *Garcinia* kola extracts and fresh honey showed a better improvement in the level of the hormone compared with the groups treated with the fresh honey and *Garcinia* kola extracts alone (group C and D). This observation could be attributed to the combined antioxidant effect of honey and *Garcinia* kola. Studies have shown that the use of a combination of antioxidants is more efficacious than the use of the individual antioxidants [1]. Erejuwa and colleagues [39] described honey as a Novel antioxidant as it is very rich in flavonoids, vitamin C and E, which are powerful antioxidants. The seeds of *Garcinia* kola contain Kolaviron (biflavonoids GB-1, GB-2 and Kolaflavone) [30]. Many investigators have reported that *Garcinia* kola seed bioactivity is linked to the presence of bioflavonoids, which are well known antioxidants [31].

5. Conclusion

The study established the ability of mixture of *Garcinia* kola extract and honey in mitigating cyclophosphamide-induced reproductive toxicity in male rats by reducing oxidative stress and attenuating endocrine disturbances in endocrine axis thereby improving Follicle stimulating Hormone, Luteinizing Hormone and testosterone levels, resulting in increased fertility potentials.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

Statement of Ethical Approval

The study protocol was approved by the Ethical and Research Committee of the University of Port-Harcourt Teaching Hospital, Niger Delta University Teaching Hospital, Okolobirir, Bayelsa State, and Federal Medical Centre Yenagoa, Bayelsa State, Nigeria. The ethical principles for medical research involving animal subjects as outlined in the Helsinki declaration in 1975 and subsequent revisions were strictly adhered to in the course of this study.

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