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Research Article

Determining the Physiological Basis of the Effects of Alligator Pepper in Pregnant Sprague Dawley Rats

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Abstract

This study was carried out to determine the changes in the fasting levels of some reproductive and metabolic hormones and glucose in pregnancy, that are due to the effect of saline extract of Alligator pepper, with the aim of determining the probable physiological mechanisms for previously reported bioactive effects. This was a controlled cross sectional intervention study. Forty-five (45) pregnant Sprague Dawley rats were allocated into experimental groups B, C, and D and 15 non-pregnant Sprague Dawley rats were allocated to the control group A. The rats in groups A and B were injected with 13.3ml/Kg body weight of normal saline intra-peritoneally while the rats in groups C and D were injected intra-peritoneally with 6.7mg/Kg body weight and 13.3mg/kg body weight of saline extract of Alligator Pepper respectively on day 4 of conception. Fasting blood glucose levels and fasting serum levels of insulin, progesterone, estradiol and prolactin were estimated on days 7, 14, and 21 of pregnancy. Observed differences between control and experimental groups were subjected to tests of significance of difference of means and $p<0.05$ was considered significant. The findings in this study were significantly increased fasting blood glucose levels in all trimesters of pregnancy, reduced fasting serum insulin level in first trimester of pregnancy, reduced fasting serum progesterone levels in first and third trimesters of pregnancy, increased fasting serum estradiol levels in first and second trimesters of pregnancy and reduced serum prolactin level in the third trimester of pregnancy in Sprague Dawley rats.

Keywords: Physiological Basis; Effects; Alligator Pepper; Pregnancy; Sprague Dawley Rats

Introduction

Aligator pepper (*Aframomum melegueta*) is a species in the ginger family, Zingiberaceae. It is commonly known as Guinea pepper or melegueta pepper. It is a herbaceous perennial plant native to swampy habitats along West African coasts. Its trumpet-shaped purple flowers develop into 5 to 7cm long pods containing numerous small reddish brown seeds. It is a widely used spice in several parts of the world [1]. Also known as Grains of paradise, it contains pungent, aromatic ketones such as 6-paradol, 6-gingerol and 6-shogaol [2]. This is in addition to sesquiterpenes and non-terpenoids.

Some of the reported bioactive effects include abortifacient properties in pregnant Sprague-Dawley rats [3], attenuation of gestational weight gain in pregnant Sprague Dawley rats [4], reduced litter size of pregnant Sprague Dawley rats [4] and hypoglycemic effects in non-diabetic and alloxan induced diabetic male albino rats [5].

The aim of this study was to determine the metabolic and hormonal changes that may explain the action of Alligator pepper in inducing first-trimester abortion [3], attenuation of gestational weight gain , reduction of litter size in pregnant Sprague Dawley rats [4], and anti-lactogenic activities as reported by

Ebong *et al* [5].

The objectives of the study were to:

• determine the changes in fasting levels of glucose, insulin, progesterone, estradiol, and prolactin in developing normal pregnancy in Sprague Dawley rats with respect to first, second and third trimesters.

• determine the effects of saline extract of alligator pepper on fasting levels of reproductive hormones namely estradiol, progesterone and prolactin during pregnancy in Sprague Dawley rats.

• determine the effect of a saline extract of alligator pepper on fasting levels of blood glucose and insulin levels in pregnancy in Sprague Dawley rats.

• elucidate the mode of action of alligator pepper in inducing attenuation of gestational weight gain, litter size reduction, anti-lactogenic effect and first-trimester abortion in Sprague Dawley rats.

Materials and Methods

Experimental Animals

Sixty female Sprague Dawley rats, each weighing between 140 and 160 grams, and 45 male Sprague Dawley rats of the same strain and of proven fertility were purchased from the Animal House of Ambrose Alli University, Ekpoma, Edo State Nigeria. They were certified by the curator to be at least six months old.

Materials

These included Grower's Mash (product of Grand Cereals Ltd, a subsidiary of UAC Ltd), and dry seeds of alligator pepper. Other materials were aluminum plates, drinking trough, beam balance, top loading balance, normal saline, calibrated glass jar, glass funnel and filter paper, beaker, syringes and needles, plastic pipette, glass slides and cover slips, microscope, glucometer, and glucometer test strips. (ACCU-CHEK; Roche) and hormone assay kits for Insulin, Progesterone, Estradiol and Prolactin produced by DRG instruments, Marburg, Germany.

Acclimatization

Sixty (60) female and 45 male Sprague Dawley rats, certified to be of proven fertility, were placed in separate cages and acclimatized for a period of two weeks. Their beddings, which were made of saw dust, were changed daily. During this period, all rats were fed with growers' mash and water *ad libitum*. They were weighed weekly throughout the duration of the study.

Research Design

This study was a controlled cross-sectional intervention study, consisting of two experiments, which ran concurrently. The first experiment was to determine the changes in fasting serum levels of insulin, progesterone, estradiol, prolactin and blood glucose in pregnant Sprague Dawley rats, on days 7, 14 and 21 of pregnancy while the second experiment was to determine the modifications made by saline extract of alligator pepper on fasting serum levels of insulin, progesterone, estradiol, prolactin and blood glucose in pregnant Sprague Dawley rats on days 7, 14 and 21 of pregnancy.

Research Method

Forty-five (45) out of the 60 female Sprague Dawley rats were randomly allocated into cages containing male Sprague Dawley rats and were allowed to remain in the cages, as monogamous pairs, for three days to enable mating to occur. The female rats that were not mated were allocated to group A while the 45 mated female rats were separated from the male rats after mating was confirmed to have occurred, placed in separate maternity cages and randomly allocated into groups B, C, and D so that each group contained 15 female rats.

Testing for Evidence of Successful Mating

To check for evidence of successful mating, the vagina was examined every morning, and vagina secretions were obtained with plastic pipettes and examined for the presence of sperm cells. In addition, the vagina and the floor of the cages were observed to check for the presence of cornified plug. The presence of sperm cells or the availability of cornified plug in the vagina or on the floor of the cage indicated successful mating, and this was regarded as day-1 of gestation[7].

Preparation and Quantification of the Extract

The extract of alligator pepper was prepared as follows: 500mls of normal saline was placed in a calibrated glass jar. Thereafter, 500mg of granulated alligator pepper was poured into the glass jar containing 500ml of normal saline and mixed thoroughly. The mixture was allowed to stand for 2 hours after which it was filtered through a glass funnel containing a filter paper into a beaker as was done in a previous study [4]. Since 500mg of granulated alligator pepper was mixed with 500ml of normal saline, each ml of filtrate or saline extract contained an equivalent of 1mg of granulated alligator pepper.

Allocation into sub-groups

In order to have separate rats for fasting blood glucose measurements and hormonal assays for days 7, 14 and 21 of pregnancy, the rats in each of the groups were allocated into subgroups so that groups A, B, C, and D then had three subgroups each namely A_1, A_2, A_3; B_1, B_2, B_3; C_1, C_2, C_3; and D_1, D_2, D_3 respectively. There were 5 rats in each of the subgroups. Fifteen non-pregnant rats in group A and 15 pregnant female rats in

Group B were injected with 13.3ml/Kg body weight of normal saline intra-peritoneally on day 4 of the conception of the pregnant rats in group B. The pregnant female rats in groups C and D were injected intra-peritoneally with 6.7mg/Kg body weight and 13.3mg/ kg body weight of a saline extract of Alligator Pepper respectively on day 4 of conception.

Measurement of Fasting Blood Glucose

After an overnight fast, the tails of the rats in subgroup A_1, B_1 C_1 and D_1 were pricked with a sterile lancet and the exuding blood was immediately placed on the glucometer test strip (Accu Chek, Roche) inserted in the glucometer. Results were then read off the glucometer (Accu Chek, Roche). This was done for the rats in the control group A_1, B_1 C_1 and D_1 on day 7. Similar procedure was also carried out for the rats in groups A_2, B_2, C_2, D_2; and A_3, B_3, C_3, D_3 on days 14 and 21 respectively.

Collection of Blood Sample and Hormonal Assay

Soon after the measurement of fasting blood glucose for each subgroup, the rats were euthanized by cervical dislocation under chloroform anesthesia and blood was immediately collected by ventricular puncture as described by Mojekwu *et al*. [6]. After clotting, serum was separated and was kept at -20ºC until used for analysis for serum insulin, progesterone, estradiol, and prolactin. The serum samples were assayed for insulin, progesterone, estradiol and prolactin using enzyme-linked immunosorbent assay kits produced by DRG Instruments GmbH, Marburg, Germany.

Statistical Analysis

The results of the measurements made were collated and means and standard errors of means were calculated and cross-tabulated. Bar charts with error bars were designed to show the difference between mean values of the control and experimental groups. Observed differences between control and experimental groups were subjected to tests of significance of the difference of means using Daniel Soper's free software for calculating One Way Analysis of Variance (ANOVA) and Student's t-test at 5% level of significance [8]. P value < 0.05 was considered significant.

Results and Discussion

When compared to non-treated pregnant rats, fasting blood glucose increased significantly ($p < 0.05$), on days 7, 14 and 21 of pregnancy (figure 1) while fasting serum insulin levels decreased significantly ($p < 0.05$) on day 7 in rats treated with 6.7mg/Kg body weight of the extract. However, there was a significant increase in the fasting serum insulin level ($p < 0.05$) on day 21with both doses of the extract (Figure 2). There was a decrease in fasting serum progesterone level on days 7, 14 and 21 of conception in rats treated with both doses of the alligator pepper extract. These decreases were significant ($p < 0.05$) on

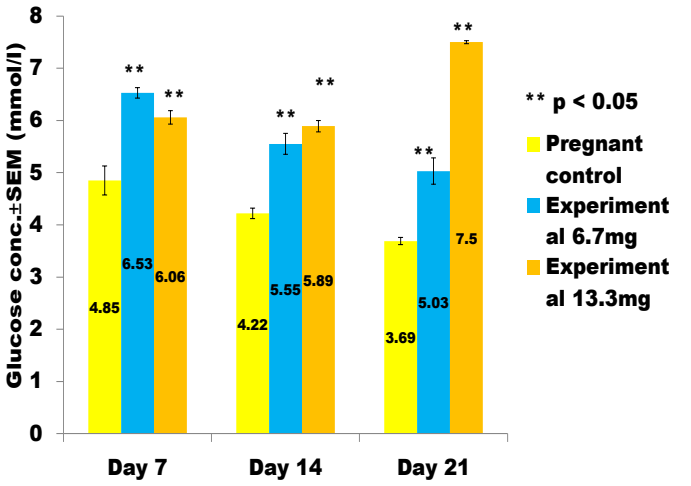

Figure 1. Effect of different doses of Saline Extract of Alligator Pepper on Fasting Blood Glucose level in Pregnant Sprague Dawley Rats.

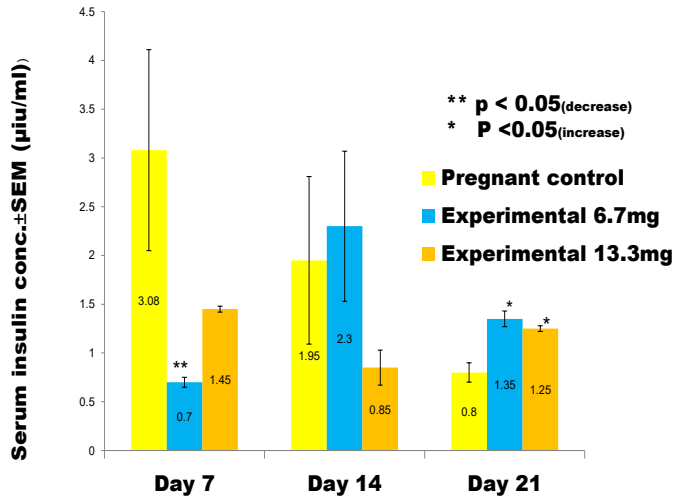

Figure 2. Effect of Different Doses of Alligator Pepper on Fasting Serum Insulin level in Pregnant Sprague Dawley Rats.

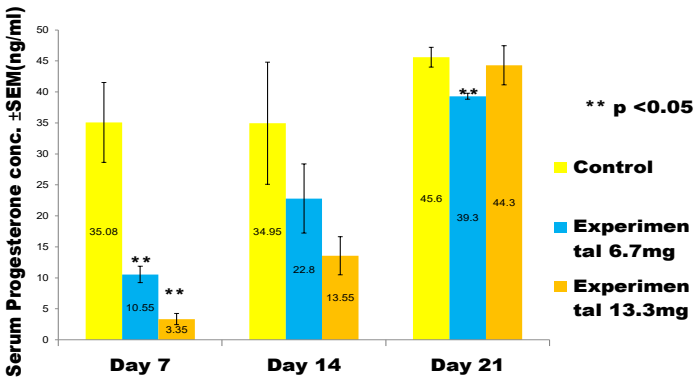

Figure 3. Effect of Different Doses of Saline Extract of Alligator Pepper on fasting serum progesterone level in Pregnant Sprague Dawley Rats.

day 7 with both doses and day 21 with 6.7mg/Kg body weight of the extract only (Figure 3). There was a dose-dependent increase in fasting serum estradiol levels on days 7 and 14 of conception, which was significant ($p < 0.05$) on days 7 and 14 with the lower dose and on day 7 only with the higher dose of Alligator pepper extract (Figure 4).

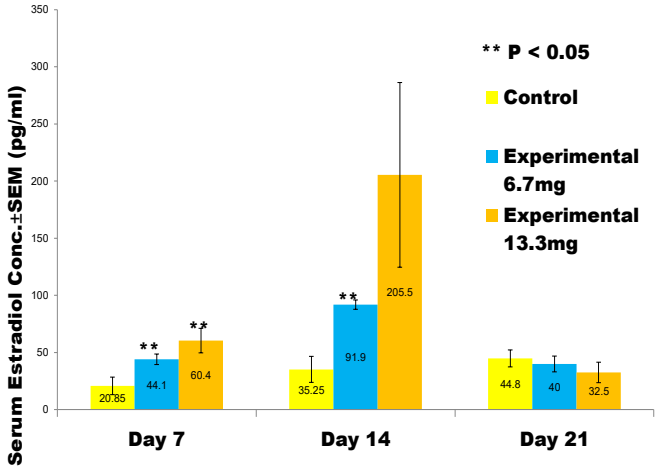

Figure 4. Effect of Different Doses of Saline Extract of Alligator Pepper on Fasting Serum Estradiol level in Pregnant Sprague Dawley Rats.

Fasting serum prolactin level decreased significantly ($p < 0.05$) on day 21 with both doses of Alligator pepper extract (Figure 5).

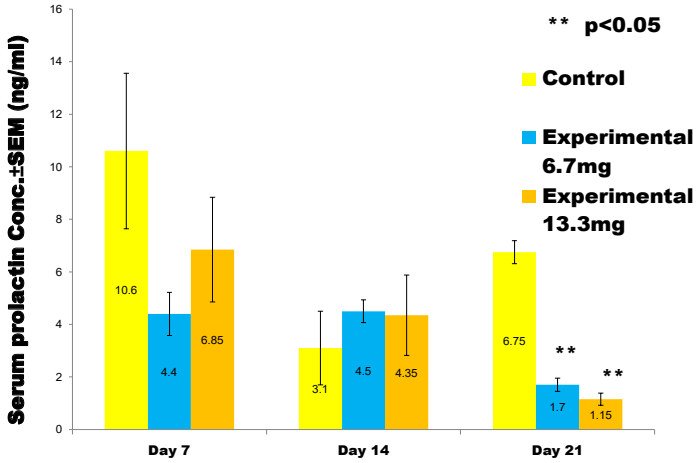

Figure 5. Effect of Different Doses of Saline extract of alligator pepper on fasting serum prolactin level in Pregnant Sprague Dawley rats.

Discussion

This study was carried out to explain the probable physiological mechanisms underlying the effects of alligator pepper in pregnant Sprague Dawley rats found in an earlier study by Inegbenebor et al. [3,4]. These effects were the first-trimester abortifacient action, attenuation of gestational weight gain, reduction in litter size and anti-lactogenic effect. Therefore, the doses of a saline extract of alligator pepper used in this experiment were some of the doses that caused attenuation of gestational weight gain and reduced litter size in the earlier study by Inegbenebor et al. [4].

Normal saline, an isotonic solution was used as the solvent 'carriage' medium in order to avoid osmotic disturbances due to hypotonic distilled water. It should be noted that normal saline is often used as a carriage medium for parenterally administered drugs. In order to avoid circulatory overload, it was noted that the blood volume of a rat is calculated as ((0.6*body weight in grams) +0.77) ml and that a 150-gram rat contained 9.77ml of blood. This implies that the maximum intraperitoneal dose of 13.3ml/Kg body weight of saline extract in a 150g rat was 2ml and this was only 20.5% of blood volume. It was also noted that 2ml of normal saline contained much less than the recommended daily water intake of 25-35ml/kg body weight and 70mmol of sodium [9]. It is known that 3-5ml of fluid can be safely injected intra-peritoneally into a rat [10].

In this study, fasting blood glucose decreased with advancing pregnancy. In pregnant rats treated with 6.7 and 13.3mg/Kg body weight of a saline extract of alligator pepper, there was a significant increase in fasting blood glucose ($p < 0.05$) in all trimesters of pregnancy. This was not consistent with a previous study by Mojekwu et al., [6], who found a non-significant decrease in fasting blood sugar using similar doses of an extract from the leaves of alligator pepper. However, leaves of alligator pepper do not contain the same constituents as the seeds of alligator pepper. While 20 constituents (mainly mrytenyl acetate (29.06%), isolimonene (19.47%) and γ-elemene (8.84%)) were identified in the essential oil of the leaves of alligator pepper, only 9 constituents (mainly α-caryophyllene (48.78%), β-caryophyllene (32.50%) and linalool (5.40%)) were identified in the seeds of alligator pepper in a recent study by Owokotomo et al., [11]. Furthermore, the difference in the dietary fiber content of the growers' mash used in both studies might offer an additional explanation for the discrepancy in results. The growers mash (produced by Grand Cereals Ltd, a subsidiary of UAC Ltd) used in this study had a dietary fiber content of 7%, while the brand used in Mojekwu's study was pelletized growers mash which had a dietary fiber content of 10% [6]. Since dietary fiber inhibits the digestion of food and reduces the absorption of post-digestion glucose [12], it is expected that more glucose will be absorbed into the circulation in this study, when compared to Mojekwu's study. Moreover, the growers mash by Grand cereals, which was used in this study, had higher protein content. High protein intake increases glucose output by the liver due to gluconeogenesis [13]. This could also account for the higher fasting glucose levels found in this study.

The outcome of pregnancy is related to the physiological and metabolic changes in pregnancy [14]. Insulin levels determine

the utilization of nutrients in the growth and development of the mother and fetus. Insulin is known to facilitate glucose uptake and weight gain [15]. It facilitates glucose and amino acid transport across membranes to fat cells, muscle, bone, kidney, hypothalamus and the fetal compartment, where it has various actions. In the fat cell, insulin facilitates lipogenesis. The end products of insulin enhanced glycolysis are alpha-glycerophosphate and free fatty acids. Free fatty acid is esterified by alpha-glycerophosphate to form triglyceride thereby increasing fat mass. Insulin enhances protein anabolism by facilitating amino acid uptake and protein synthesis while depressing gluconeogenesis [16]. This effect increases the size of muscles [17]. Insulin-like growth factor 1 (Igf1) increases longitudinal bone growth by facilitating chondrocyte hypertrophy [18]. In the kidney, insulin causes sodium and water retention by activating epithelial sodium channel (ENaC channel) [19]. High level of insulin inhibits leptin, depressing satiety and facilitating the development of obesity [20]. In the fetal compartment, there is also increased fat mass, increased muscle mass and longitudinal bone growth. Therefore, when there is excessive secretion of insulin in pregnancy as is found in insulin resistance and maternal over-nutrition, the result is excessive gestational weight gain and large litters.

However, when there is a relative insulin deficiency as was found in alligator pepper treated rats, there is dis-inhibition of triglyceride lipase, which enhances lipo-oxidation [17]. Furthermore, there is a decrease in the formation of alpha-glycerophosphate, which is needed for the esterification of free fatty acids to form triglyceride and increase fat mass. In addition, there is diminished sodium and water retention. There is also a diminished protein synthesis, the result of which is reduced muscle mass. Leptin is also uninhibited leading to satisfaction with a normal quantity of food. The consequent diminished fat mass, diminished salt and water retention and reduced muscle mass lead to attenuation of gestational weight gain, reported in an earlier study by Inegbenebor et al., [4].

In the presence of relative insulin deficiency found in alligator pepper treated pregnant Sprague Dawley rats, there is a diminished transfer of glucose into the fetal compartment, diminished insulin response from the fetal pancreas with a consequent reduction in fat mass, muscle size and longitudinal bone growth leading to a reduction in litter size.

In this study, there was an increasing fasting serum progesterone level as pregnancy advanced. At least 10ng/ml of progesterone is required for the sustenance of pregnancy [21]. In this study, fasting serum progesterone level was higher than 10ng/ml in non-treated pregnant rats in all trimesters of pregnancy. Though alligator pepper treated pregnant Sprague Dawley rats had significantly diminished progesterone levels with the doses applied, none of the rats aborted. However, a higher dose of 333mg/kg body weight caused first trimester abortion in a previous study [3].

The abortifacient effect was probably due to caryophyllene, a constituent of alligator pepper and a type 2 cannabinoid receptor agonist, which inhibited the synthesis of testosterone in a previous study [22] . This is probably due to the effect of Caryophyllene on 3 β hydroxysteroid dehydrogenase, an enzyme, which converts pregnenolone to progesterone, a step in the synthetic pathway of testosterone [23].

A significant increase in fasting serum estradiol level was also observed in this study ($p < 0.05$). This rise was probably due to the effect of one of the constituents of alligator pepper on an enzyme in other components of the fetoplacental unit since the placenta lacks 17 α hydroxylase activity [24]. This implies that conversion of C21 to C19 steroids (the latter being the immediate and obligatory precursors of estrogens) is not possible. However, there is an exceptionally high capacity of the placenta to convert C19 steroids (e.g dehydroepiandrosterone and its sulfate) to estrone and estradiol. The conversion of dehydroepiandrosterone sulfate to estradiol requires four key enzymes namely: sulphatase, which converts dehydroepiandrosterone sulfate to dehydroepiandrosterone; 3β hydroxysteroid dehydrogenase, which converts dehydroepiandrosterone to androstenedione; cytochrome P450 aromatase, which converts androstenedione to estrone and 17β hydroxysteroid dehydrogenase, which converts estrone to estradiol. The principal cellular location of the four enzymes is the syncytiotrophoblast [25]. Beta-Caryophyllene, a major constituent of alligator pepper is known to significantly increase serum estradiol levels in male Wistar Han rats [26].

In this study, there was a significant decrease in serum prolactin level in treated pregnant rats on day 21($p < 0.05$) when compared to non-treated pregnant rats. This was consistent with previous findings by Ebong *et al* [5]. Systemically administered cannabinoids such as caryophyllene enhance release of dopamine in the nucleus accumbens due to enhanced firing of the mesolimbic dopaminergic neurons [27]. Dopamine is prolactin inhibitory hormone (PIH) [28]. Enhanced release of dopamine (PIH) inhibits prolactin release from the lactotrophs of the anterior pituitary gland.

The hormones mentioned in this project are not exhaustive. Human placental lactogen, cortisol, adrenalin, growth hormone and leptin all play a part in blood glucose homeostasis. Cytokines such as adiponectin, Tumor Necrotic Factor alpha (TNFα) and Resistin play various roles in insulin resistance [15]. This was a cross-sectional intervention study. A longitudinal study would have given more reliable results but could not be done with rats that must be euthanized to get adequate quantity of blood sample. The disparity in some of the hormonal levels could be due to the fact that samples were collected from different rats.

Conclusion

In conclusion, intraperitoneally administered saline extract of

alligator pepper significantly ($p < 0.05$)

– increased fasting blood glucose in all trimesters of pregnancy,

– reduced serum insulin in the first trimester of pregnancy,

– reduced serum progesterone in the first and third trimester of pregnancy,

– increased serum estradiol in first and second trimesters of pregnancy and

– reduced serum prolactin levels in the third trimester of pregnancy in Sprague Dawley Rats

The decrease in insulin level in alligator pepper treated Sprague Dawley rats was responsible for the attenuation of gestational weight gain and litter size reduction while the decrease in progesterone level was responsible for the abortifacient action. The decrease in prolactin was responsible for the anti lactogenic activity.

The most probable active ingredient of Alligator pepper was caryophyllene, a type 2 beta receptor cannabinoid.

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Conflict of Interest

None

Contribution

U. I carried out the research and wrote this article under the supervision of Professor M. I. Ebomoyi and Professor L. F. O. Obika.

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