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Hormonal and electrolyte assessment on the effect of garlic (*Allium sativum*), Vitamin C and E in tramadol induced toxicity in female Wistar rats

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Abstract
The ameliorative antioxidant effect of vitamin C, E and garlic on tramadol induced toxicity was studied in wistar rats. Thirty five (35) rats were used and divided into five study groups with seven rats each. There was significant decrease in the body weight of the negative control group compared with the positive control group. Also, there was a significant increase in the body weight indices of antioxidant vitamins C, E and garlic treated groups when compared with the negative control at ($P < 0.05$). The organ weight of the liver in the negative control group increased significantly when compared to the weight of the liver in the positive control group. This reveals the metabolic changes in the liver as the site of drug metabolism which resulted in the inflammation of the liver. The weights of the heart and kidney also showed similar differences in the graphs. There was significant decrease in the Na^+, K^+, level of the negative control compared to the positive control group at ($P<0.05$). The total level of antioxidants vitamin C, E and garlic increased significantly when compared to the negative control group ($P<0.05$). The level of K^+ in the negative control group reduced significantly when compared to the positive control group at ($P<0.05$) but HCO_3^- increased significantly in the negative control compared to the positive control at ($P<0.05$), in other groups (TmVC, TmVE, TmG), HCO_3^- elevated significantly compared to the control group at ($P<0.05$). The serum activities of female hormones; LH and Estrogen group showed significant decrease in the negative control group at ($P<0.05$) when compared to the positive control group at ($P<0.05$) but in the TmVC, TmVE, TmG group, LH and estrogen showed significant increase but not as the positive control group.

Keywords: tramadol, estrogen, Lutinizing hormone electrolytes, vitamin C, E and garlic

1. Introduction
Drug abuse is the recurrent use of illegal drugs, misuse of prescription and/or over the counter drugs with negative consequences. Drug abuse in Nigeria has been indicated to be on the rise in recent years. National Drug Law Enforcement Agency (NDLEA) pointed that Kano State has the highest drug abuse rates based on the number of seizures, arrests of addicts and convictions of arrested dealers (Babatunde and Abiodun, 2015) [2]. This use of hard drugs as well as misuse of prescription drugs for nonmedical purposes cut across all strata especially the youths. Such abused drugs include alcohol, tobacco, heroine, caffeine, opiates, over the counter drugs etc (Lee *et al.*, 2002) [12]. Youths are known to continue using these drugs despite the major risk behaviours with its accompanied physical and mental health complications (Hamisu *et al.*, 2014) [9]. Of growing interest is the increased demand for tramadol which is an opiate analgesic medication 2[(Dimethylamin) methyl]-1-(3-methoxyphenyl) cuclohexanol also known as tramadol is a common over the counter drug sold in pharmaceutical shops and retail medicine outlets in Nigeria. It is an opioid pain medication for the treatment of moderate to moderately severe pains. Available dosage forms include capsules, tablets and extended release formulations and injections (Brayfield, 2013) [6].
Tramadol has been shown to have high bioavailability with a range of 70-80% with peak blood levels of about 2 hours following oral administration. It is metabolized in the liver by cytochrome P450 (Abdelraouf *et al*., 2015) [1]. Bio-transformed to five different metabolites of which O-desmetyl-tramadol is the most significant and is 2-4 times more active than tramadol (Samer *et al*., 2013) [18]. By-products of tramadol are excreted through the kidneys (Abdelraouf *et al*., 2015) [1]. Tramadol is frequently been abused in Northern Nigeria by youths for lasting energy (Pharmanews, 2015) [15] and sexual performance drug (Daily Trust Nigeria, 2014). The use of this potent opioid pain medication as sexual enhancer and proposed management of premature ejaculation as proposed by (Safarinejad and Hosseini, 2006; Salem *et al*., 2008; Bar-Or *et al*., 2012; Yang *et al*., 2013) [16, 17, 3, 19] as well as the effectiveness of tramadol in the treatment of premature ejaculation as shown by (Bayoumy *et al*., 2012) [4] have given rise to this study. More so, tramadol is given to women during labour to reduce some level of pains, so this research will check the extent whether or not, affect the level of reproductive/sex hormones in female.
Antioxidants are natural or synthetic substances that may prevent or delay some types of cell damage. Antioxidants are found in many foods, including fruits and vegetables. Although oxidation reactions are crucial for life, they can also be damaging; plants and animals maintain complex

systems of multiple types of antioxidants, such as glutathione, vitamin C, vitamin A, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxides. Traditional herbal medicines, dietary foods were the main source of antioxidant for ancient peoples that protected them from the damage caused by free radicals. Antioxidants are widely used in dietary supplements and have been investigated for the prevention of diseases such as cancer, coronary heart disease and even altitude sickness. Although initial studies suggested that antioxidant supplements might promote health, later large clinical trials of antioxidant supplements including beta-carotene, vitamin A, and vitamin E singly or in different combinations suggest that supplementation has no effect on mortality or possibly increases it. These are also use in the food industry in the form of preservatives in foods and cosmetics and to prevent the degradation to rubber and gasoline.

Antioxidants are believed to play a very important role in the body defense system against Reactive Oxidative Species (ROS) Boxin *et al*., (2002) [5]. In another term antioxidant is "any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate Halliwell and Gutteridge (1995) [11]. Halliwell (2007) reported that an antioxidant is "any substance that delays, prevents or removes oxidative damage to a target molecule. Antioxidants are an inhibitor of the process of oxidation, even at relatively small concentration and thus have diverse physiological role in the body. Antioxidant constituents of the plant material act as radical scavengers, and helps in converting the radicals to Less Reactive Species (LRS), a variety of free radical scavenging antioxidants is found in dietary sources like fruits, vegetables and tea, etc. Studies demonstrate that an antioxidant-rich diet has a very positive health impact in the long run Sin *et al*., (2013) and Willis *et al*., (2009). It is a well-known fact that citrus fruits (oranges, lemons, etc.) contain a high amount of natural antioxidants, such as vitamin C. Blueberries, strawberries, grapes, plums, prunes, red beans, spinach, kale, broccoli flowers, alfalfa sprouts, and more have been proven to contain a high amount of antioxidants and have been incorporated into many dietary menus Cao *et al*., (1998) [7] and Grossman *et al*., (1994). Recent studies also suggested that fruit-like jackfruit, araticu-domato, pindo palm, and mandacaru-de-trêsquinas are good sources of vitamins C and A and phenolic compounds (Swami *et al*., (2012) and Pereira *et al*., (2013).

Vitamin E is a collective term given to a group of fat-soluble compounds first discovered in 1922 by Evans and Bishop; these compounds have distinct antioxidant activities essential for health (Niki and Traber, 2012). Vitamin E is present in fat-containing foods and, as the fat-soluble property of the vitamin allows it to be stored within the fatty tissues of animals and humans, it does not have to be consumed every day (Zingg, 2007). The vitamin E group (i.e. chroman-6-ols), collectively termed tocochromanols (divided into tocopherols and tocotrienols), includes all of the tocol and tocotrienol derivatives which qualitatively exhibit the biological activity of d-alpha-tocopherol. There are eight naturally occurring forms of vitamin E; namely, the alpha, beta, gamma and delta classes of tocopherol and tocotrienol, which are synthesised by plants from homogentisic acid. Alpha- and gamma-tocopherols are the two major forms of the vitamin, with the relative proportions of these depending on the source.

Electrolytes are minerals that have many functions in the body including maintaining water balance, helping your muscles (including your heart) contract and relax and helping transmit nerve impulses. The most common electrolytes are sodium, potassium, and chloride, which are lost in sweat along with water. Sodium regulates the total amount of water in the body and maintains the proper function of the nervous, muscular, and other systems. Potassium is responsible for regulating heartbeat and muscle function and is important in neuron function. Extreme high or low potassium levels can cause irregular heartbeat, which can be fatal. Chloride helps maintain a normal balance of body fluids.

Materials and methods

Experimental animals

Thirty five female wistar rats were obtained from the animal house of the college of medical sciences, University of Calabar, Calabar, Nigeria. The rats were assigned into five study groups of seven rats each. The rats were kept under normal laboratory conditions of temperature, light and humidity in secure iron cages. They were allowed free access to clean water and animal feed. The animals were allowed two weeks acclimatization and their weights measured before treatment commenced.

Source of drugs

Tramadol, Vitamin C, Vitamin E and Garlic were all purchased from a registered pharmacy in Benz pharmacy at Etta-Agbor road, Calabar, Cross River State, Nigeria and used for the study.

Chemicals and Equipments: The dissecting kits, kitchen scale (BS 2508 model). The cages were gotten from the animal house of medical college, university of Calabar. The sample containers, hand gloves, syringes and needles were purchased from Benz pharmacy, etta-agbor road, Calabar.

Experimental design

The grouping and treatment given to the rats in each groups are as follows;

Group A: Designated NT consisted of positive control rats without any treatment Group B: Designated Tm consisted of negative control rats administered 0.2mg of tramadol Group C: Designated TmVC consisted of rats administered 0.2mg of tramadol and 0.2ml of vitamin C Group D: Designated TmVE consisted of rats administered 0.2mg of tramadol and 0.2ml of vitamin E. Group E: Designated TmG consisted of rats administered 0.2mg of tramadol and 0.2ml of garlic

Sacrifice of the animals

At the end of the experimental period, rats in each group were fasted overnight and sacrificed under anesthesia by cervical dislocation.

Serum collection and organ extraction

After the rats have been sacrificed, 2 – 4ml of blood was collected from each rat and placed in specific sterile bottles for hormonal and electrolyte analysis The organs such as the liver, heart and kidney were carefully extracted and weighed.

Hormonal Assay

Luteinizing hormone

Principle

The LH Quantitative Test was based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay system utilizes a mouse monoclonal anti-ALPHA-FSH antibody for solid phase (microtiter wells) immobilization and another mouse monoclonal anti-β-LH antibody in the antibody enzyme (horseradish peroxidase) conjugate solution. The test sample was allowed to react simultaneously with the antibodies, resulting in LH molecules being sandwiched between the solid phase and enzyme-linked antibodies. After 45-minute incubation at room temperature, the wells were washed with water to remove unbound-labeled antibodies. A solution of TMB Reagent was added and incubated at room temperature for 20 minutes, resulting in the development of a blue color. The color development was stopped with the addition of Stop Solution and the color was changed to yellow and measured spectrophotometrically at 450 nm. The concentration of LH is directly proportional to the color intensity of the test sample (Lequin, 2005) [13].

Procedure

Desired number of coated wells was secured in the holder. 50µl of standard, specimens, and controls were dispensed into appropriate wells, 100µl of Enzyme Conjugate Reagent was dispensed into each well after which mixtures were thoroughly mixed for 30 seconds. It is very important to have a complete mixing in this setup. They were incubated at room temperature (18-25°C) for 45 minutes. The incubation mixture was removed by flicking plate contents into a waste container. The microtiter wells were rinsed and flicked 5 times with distilled or deionized water. The wells were sharply struck onto absorbent paper or paper towels to remove all residual water droplets then 100µl TMB Reagent was dispensed into each well and gently mixed for 10 seconds. They were incubated at room temperature in the dark for 20 minutes then the reaction was stopped by adding 100µl of Stop Solution to each well. Wells were gently mixed for 30 seconds. It is important to make sure that all the blue color changes to yellow color completely. The optical density was read at 450nm with a microtiter plate reader within 15 minutes (Lequin, 2005) [13].

Estrogen

Principle

The Estrogen EIA was based on the principle of competitive binding between Estrogen in the test specimen and Estrogen-HRP conjugate for a constant amount of rabbit anti-Estrogen. In the incubation, goat anti-rabbit IgG-coated wells were incubated with 25µl Estrogen standards, controls, patient samples, 100µl Estrogen-HRP Conjugate Reagent and 50µl rabbit anti-Estrogen reagent at room temperature (18-25°C) for 90 minutes. During the incubation, a fixed amount of HRP-labeled Estrogen competes with the endogenous Estrogen in the standard, sample, or quality control serum for a fixed number of binding sites of the specific Estrogen antibody. Thus, the amount of Estrogen peroxidase conjugate immunologically bound to the well progressively decreases as the concentration of Estrogen in the specimen increases. Unbound Estrogen peroxidase conjugate was then removed and the wells washed. Next, a solution of TMB Reagent was added and incubated at room temperature for 20 minutes, resulting in the development of blue color. The color development was stopped with the addition of 1N HCl, and the absorbance was measured spectrophotometric ally at 450 nm. The intensity of the color formed was proportional to the amount of enzyme present and is inversely related to the amount of unlabeled Estrogen in the sample. A standard curve is obtained by plotting the concentration of the standard versus the absorbance. The Estrogen concentration of the specimens and controls run concurrently with the standards can be calculated from the standard curve (Lequin, 2005) [13].

Procedure

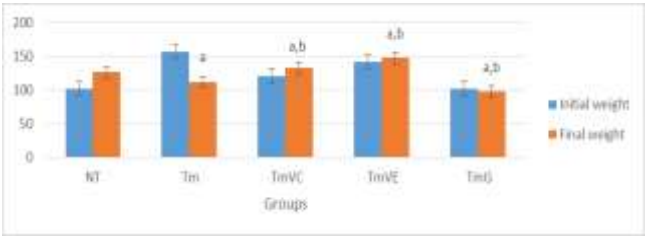
All reagents were brought to room temperature (18-25°C) before use. Desired numbers of coated wells were secured in the holder. 25µl of standards, specimens and controls were dispensed into appropriate wells after which 100µl of Estrogen-HRP Conjugate Reagent was dispensed into each well, 50µl of rabbit anti-Estrogen reagent was also dispensed into each well. Mixture was thoroughly mixed for 30 seconds. It is very important to mix them completely then they were incubated at room temperature (18-25°C) for 90 minutes. The microwells were rinsed and flicked 5 times with distilled water then 100µl of TMB Reagent was dispensed into each well and gently mixed for 10 seconds. They were incubated at room temperature (18-25°C) for 20 minutes. Reaction was stopped by adding 100µl of Stop Solution to each well. Wells were gently mixed for 30 seconds. It is important to make sure that all the blue color changes to yellow color completely. Absorbance was read at 450 nm with a microtiter well reader within 15 minutes (Lequin, 2005) [13].

Determination of electrolytes level: Ion Selective Electrodes (ISE) was used to determined the ion level.

Statistical analysis

The data will be expressed as mean value +- S.E.M (standard error of mean). All results will be the mean of 7 data samples and the statistical analysis will be carried out using students t-test.

Results



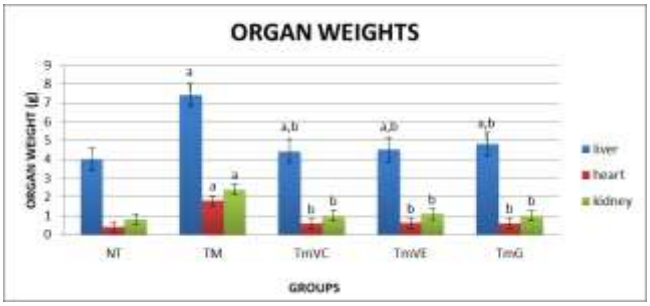
Value expressed in mean ±SEM of 7 determination

Fig 1: Effect of tramadol and antioxidants vitamin C, E and garlic interactions on the body weight.

NT: Group I, positive control, no tramadol administered
TM: Group II, negative control, only tramadol administered
TmVC: Group III, tramadol and vitamin C administered
TmVE: Group IV, tramadol and vitamin E administered
TmG: Group V, tramadol and garlic administered

a. Shows significant difference when compared with positive control at ($P<0.05$)

b. Shows significant difference when compared with negative control at (P<0.05)

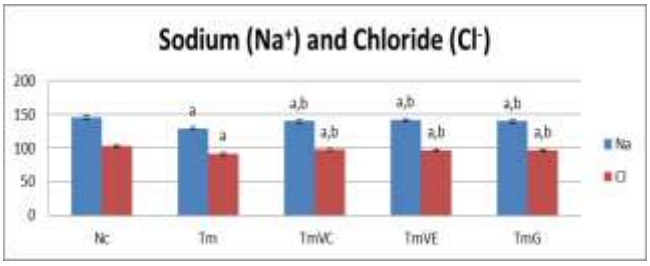


Value expressed in mean ±SEM of 7 determination

Fig 4.2: effect of tramadol and antioxidants vitamin C, E and garlic interactions on organ weight.

NT: Group I, positive control, no tramadol administered
TM: Group II, negative control, only tramadol administered
TmVC: Group III, tramadol and vitamin C administered
TmVE: Group IV, tramadol and vitamin E administered
TmG: Group V, tramadol and garlic administered

a. Shows significant difference when compared with positive control at (p<0.05)
b. Shows significant difference when compared with negative control at (p<0.05)



Value expressed in mean ±SEM of 7 determination

Fig 3: effect of tramadol and antioxidants vitamin C, E and garlic interactions on Sodium and Chloride.

NT: Group I, positive control, no tramadol administered
TM: Group II, negative control, only tramadol administered
TmVC: Group III, tramadol and vitamin C administered
TmVE: Group IV, tramadol and vitamin E administered
TmG: Group V, tramadol and garlic administered

a. Shows significant difference when compared with positive control at (p<0.05)
b. Shows significant difference when compared with negative control at (p<0.05)

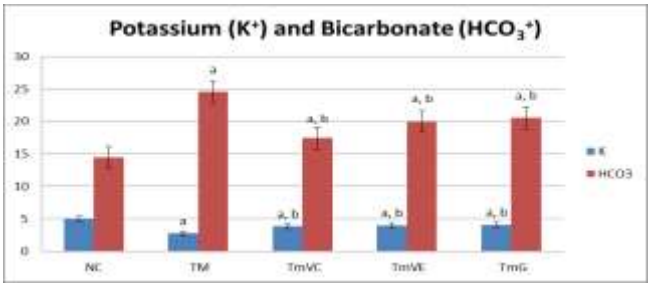


Fig 4: Effect of tramadol and antioxidants vitamin C, E and garlic interactions on Potassium and Bicarbonate.

NT: Group I, positive control, no tramadol administered
TM: Group II, negative control, only tramadol administered
TmVC: Group III, tramadol and vitamin C administered
TmVE: Group IV, tramadol and vitamin E administered
TmG: Group V, tramadol and garlic administered

a. Shows significant difference when compared with positive control at (p<0.05)
b. Shows significant difference when compared with negative control at (p<0.05)

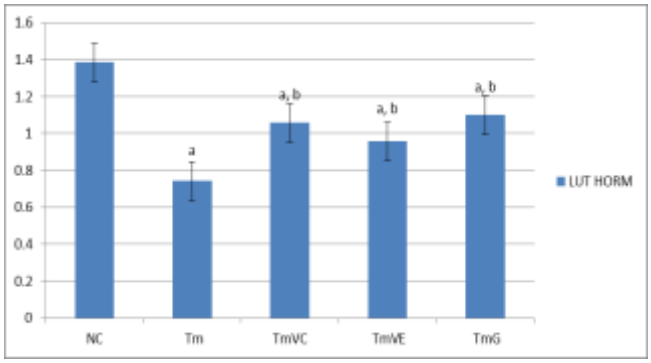


Fig 5: Effect of tramadol and antioxidants vitamin C, E and garlic interactions on Luteinizing hormone.

NT: Group I, positive control, no tramadol administered
TM: Group II, negative control, only tramadol administered
TmVC: Group III, tramadol and vitamin C administered
TmVE: Group IV, tramadol and vitamin E administered
TmG: Group V, tramadol and garlic administered

a. Shows significant difference when compared with positive control at (p<0.05)
b. Shows significant difference when compared with negative control at (p<0.05)

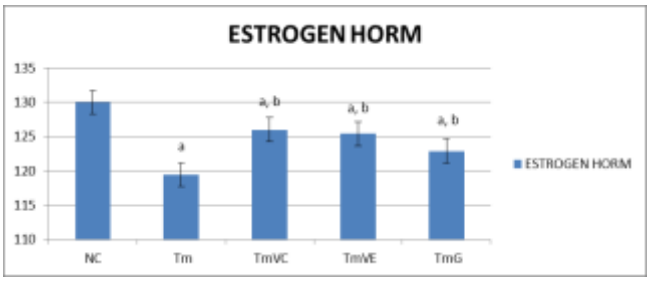


Fig 6: Effect of tramadol and antioxidants vitamin C, E and garlic interactions on Estrogen hormone.

NT: Group I, positive control, no tramadol administered
TM: Group II, negative control, only tramadol administered
Tm VC: Group III, tramadol and vitamin C administered
Tm VE: Group IV, tramadol and vitamin E administered
Tm G: Group V, tramadol and garlic administered

a. Shows significant difference when compared with positive control at (p<0.05)
b. Shows significant difference when compared with negative control at (p<0.05)

Discussion

This research work was carried out to evaluate and compare the effects of Vitamin C, vitamin E and garlic on the hormonal and electrolyte level of Tramadol induced Wistar rats. Tramadol, antioxidant vitamins; garlic, vitamin C and vitamin E were administered to the animals concomitantly for a period of twenty eight (28) days.

There was a significant (p<0.05) decrease in the body weight of the negative control group in which tramadol

toxicity was induced. The final body weight of the rats in this group dropped significantly from the initial body weight and also when compared to the final body weights of the rats in the positive control group at ($p<0.05$). This is because one of the effects of tramadol induced toxicity is a lack of appetite which leads to loss of weight. However, there was a decrease in the final weights of the other groups (which includes the TmVC group, the TmVE group and the TmG) when compared to the negative control group which shows the antioxidant activities of vitamin C, E and garlic to mop up the free radicals produced by tramadol induced toxicity.

The weight of the liver in the negative control group rose significantly ($p<0.05$) when compared to the weight of the liver in the positive control group. This shows the stress undergone by the liver as the body strives to convert the tramadol through the ADME (absorption, digestion, metabolism and excretion) pathway to a less harmful substance, this results in the inflammation of the liver. The weight of the liver also rises in the other groups significantly compared to the positive control group, however the rise is not pronounced as that of the negative control group and when these other groups are compared with the negative control group at ($p<0.05$) there is a significant decrease, once again highlighting the effects of the antioxidants vitamin C, E and garlic. The weights of the heart and kidney also showed similar variations in the graphs. This is because all three organs which include the liver, kidney and heart are affected to an extent by the tramadol induced toxicity.

There was a significant decrease in the Na^+, K^+, level of the negative control compared to the positive control group at ($P<0.05$). The total level of Na^+, K^+, in antioxidants vitamin C, E and garlic increased significantly when compared to the negative control group ($P<0.05$). The level of K^+ in the negative control group reduced significantly when compared to the positive control group at ($P<0.05$) but HCO_3^- increased significantly in the negative control compared to the positive control at ($P<0.05$), in other groups (TmVC, TmVE, TmG), HCO_3^- elevated significantly compared to the control group at ($P<0.05$). The serum activities of female hormones; LH and Estrogen group showed significant decrease in the negative control group at ($P<0.05$) when compared to the positive control group at ($P<0.05$) but in the TmVC, TmVE, TmG group, LH and estrogen showed significant increase and may suggest the ameliorative potentials of these antioxidant on female reproductive hormones.

Conclusion

Vitamin C, E and Garlic had significant effects on the hormonal and electrolytes level in wistar rats under tramadol induced toxicity. The antioxidants succeeded in ameliorating the effect of tramadol on the electrolyte and hormonal level during tramadol induced toxicity.

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