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Changes in Plasma Calcium and Inorganic Phosphate Concentration in Combined Decoction of Citrus Aurantifolia and Camellia Sinensis Treated Rats

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***Abstract*— In this study, the effects of the decoction of lime and lipton on plasma concentrations of calcium, inorganic phosphate and weight of rats were evaluated. Twenty four (24) healthy female albino rats of Wistar strain with an average weight of 150g, were randomly placed into two (2) test and control groups of twelve (12) rats each. Blood samples were collected and analyzed for plasma calcium and phosphate concentration. Results revealed that decoction of lime and lipton tea caused a significant increase in plasma calcium concentration and non-significant reduction in inorganic phosphate concentration. The increase in the weight of rats apparently due to the decoction was not significant. Histological studies showed that the decoction of lime and lipton tea has a toxic effect on the liver but was not toxic to the kidney and the heart. Therefore the decoction could be useful in increasing the bone mineral content and bone mineral density of tibia which could be beneficial in preventing osteoporosis even in humans.**

***Index Terms*— Citrus aurantifolia (lime), Camellia sinensis (Lipton tea), plasma Calcium and inorganic phosphorus.**

I. INTRODUCTION

Man uses plants in different ways according to his needs, particularly as food and medicine. Among the entire flora, 35,000 to 70,000 species have been used for medicinal purposes.[1] In view of the onset of modern medicine, the traditional plant medicine has taken a back seat although they still play vital remedial roles more in developing countries than in the developed countries. The disadvantage of the use of modern drugs in view of their short and/or long term side effects has made the scientists to develop or design and formulate the newer drugs with negligible side effects for treatment of diseases from medicinal plants. This has accelerated the effort to understand the scientific basis underlying the effective control and treatment of the chronic diseases by medicinal herbs.[1]

Citrus fruits, which are one of the most important commercial crops grown in all continents of the world ,have received attention not only for their nutritional properties but also for their medicinal value. Some *Citrus* species have indeed a broad spectrum of biological activities, including antibacterial, antioxidant, antilarvae, antidiabetic and anti-inflammatory activities.[2,3,4,5,6]*Citrus* fruits are rich sources of ascorbic acid and other bioactive compounds such as coumarins, carotenoids, limonoids and flavonoids (in particular, polymethoxylated flavones and flavanones). *Citrus* flavonoids have been found to possess a wide range of activities. This class is known, for example, to act asfree radical scavengers, to modulate enzymatic activities, andto inhibit cellular proliferation as well as possessing antibiotic,anti-allergenic, anti-diarrhea, anti-ulcer, and anti-inflammatory activities.[7]Thefindings of a recent study suggests that *C. aurantifolia* essential oil could play a role for the treatment of drug-induced obesity,since it affects food intake as well as a diverse array of processes involved in energy expenditure and fuel utilisation, all of which suppress weight gain.[8]Inaddition, *C. aurantifolia* peel essential oil, juice and extract were reported to have protective effects against osteoporosis.[9]

After water, tea is the most popularly consumed beverage worldwide with a per capita consumption of 120 ml/day. All tea are produced from the leaves of the tropical evergreen *C. Sinensis*. The leaf extract had been reported as potential antioxidant and potent remedy for cancers in traditional medicines due to the presence of some metabolites such as flavonoids, alkaloids and terpenoids in the leaf extracts.[10] Its antioxidant, anti-carcinogenic, antibacterial, anti-obesity, anti-diabetic and anti-osteoporotic activities has also been reported. [10,11,12,13,14,15]

Calcium is the most abundant mineral in the human body. The average adult body contains in total approximately 1 kg, 99% in the skeleton in the form of calcium phosphate salts and 1% in plasma. Of the 1% in plasma, 35-50% is protein-bound, 5-10% is in the form of complexes with organic acids and phosphates and the remainder (50-60%) is ionized. Calcium metabolism refers to the movements and regulation of calcium ions (Ca^{2+}) in and out of various body compartments, such as the gastro-intestinal tract, the blood plasma,the extracellular and intracellular fluids, and bone tissue. An important aspect of calcium metabolism is plasma calcium homeostasis; the regulation of calcium ions in the blood plasma within narrow limits.[16]In this process, bone tissue acts as a calcium storage center for deposits and withdrawals as needed by the blood, via continual bone remodeling. Derangement of this mechanism leads to hypercalcemia or hypocalcaemia, both of which can have

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consequences in health. The level of the calcium in human's plasma is regulated by calcitonin and parathyroid hormone (PTH); calcitonin is released by the thyroid gland when its plasma level is above its set normal point (in order to lower calcium level); PTH is released by the parathyroid glands when calcium level falls below set point (in order to raise it).[17]

Inorganic phosphate has a number of functions ranging from several enzymatic reactions to components of energy in form of sodium/potassium adenosine triphosphatase, 2,3,DPG in the respiratory pathway and a major component of bone in association with calcium salt. The regulation of phosphate is mainly in association with calcium by parathyroid hormone,calcitonin and calcitriol(vitamin D).When the plasma phosphate level is low or reduces, calcitonin secretion is inhibited and PTH secretion is stimulated, resulting in phosphate being removed from bone to rapidly correct the plasma phosphate level. The high plasma PTH levels inhibit calcium loss via the urine while stimulating the excretion of phosphate ions via that route. They also stimulate the kidneys to manufacture calcitriol (a steroid hormone), which enhances the ability of the cells lining the gut to absorb both calcium and phosphate from the intestinal contents into the blood, by stimulating the production of calbindin in these cells. The PTH stimulated production of calcitriol also causes calcium to be released from bone into the blood, by the release of RANKL (a cytokine, or local hormone) from the osteoblasts which increases the bone resorptive activity by the osteoclasts. These are, however, a relatively slow processes.[16]

There are only a few studies if at all on the combined decoction of lime and tea but there are a number of previous studies, [9,15] on the effects of decoction of lime or tea which reported improved levels of plasma calcium and inorganic phosphate content thereby reducing bone loss which in effect protected against osteoporosis in ovariectomized rat model.

The aim of this study therefore was to examine the combined effect of the decoction of lime and lipton tea on plasma calcium and inorganic phosphate concentration in albino rats in our environment.

II. MATERIALS AND METHODS

Materials

Chemicals/Reagents

All chemical reagents used were of analytical grade as manufactured by Randox laboratory Ltd in England. The kit was supplied by Pyrex reagent a commercial supplier.

Experimental Animals

Twenty four (24) healthy female albino rats of wistar strain aged 3 weeks and with an average weight of 150g were used in this study. They were obtained from the animal house of Vetcare pharmaceuticals, Port-Harcourt and maintained under standard laboratory conditions. The animals were acclimatized for one week and during this period were fed with pelleted growers mash and clean distilled water.

Feeds

Rats were fed with pelleted growers mash throughout the duration of the experiment.

The Lime

Lime specimen(*Citrus aurantifolia)* and the lipton tea(*Camellia sinensis)* were purchased from, Amassoma, market, Bayelsa state.

Methods

Preparation of Extracts

Two (2) limes were thoroughly washed with clean water and cut into 2 apiece. They were then introduced into a 500ml beaker containing 200 ml of pure water (distilled water) and a bag of lipton tea. The mixture was heated/boiled for 20minutes. The decoction was allowed to cool down under room temperature and sieved with the aid of a sterile cheese cloth. The extract was stored in a clean bottle and kept in the refrigerator at 4^{o}C until used. This procedure was repeatedly done to have a routine fresh sample.

Administration of Extract

The decoction of lime and lipton tea (experimental sample) was administered to the experimental animals orally (P.O) using a gavage at 2ml/Kg body weight of rat for three times daily (8 hourly).

Ethical Clearance

The entire experimental protocol was performed in accordance with the Institutional Animal Ethical Committee (IAEC), in line with the directions of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) in Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria.

Experimental Design

The twenty four (24 healthy) female albino rats of Wistar strain after one week acclimatization were randomly distributed into two groups containing 12 rats each.

Group 1 served as control and received pelleted growers mash and distilled water throughout the experiment.

Group 2 served as the test and was administered experimental sample at 2ml/Kg body weight and also fed with pelleted growers mash and clean distilled water throughout the experiment.

Blood Collections and Analysis

Blood collected was immediately subjected to centrifugation at 3000Xg for 20minutes to obtain the plasma. Analysis was carried out immediately after centrifugation.

Calcium Assay

Principle

Calcium ions form a violet complex with O-Cresolpthaleincomplexone in an alkaline medium to form a purple colored complex. The absorbance of this complex is proportional to the calcium concentration in the sample.

Procedure

Reagent blank	Standard	Sample
Sample	-	- 20µl
Standard	-	20µl -
Distilled water	20µl	- -
Reagent	1ml1ml1ml	

Mix and incubate at room temperature for 5mins, measure the absorbance of the standard and sample against the reagent blank at 570nm.

CALCULATION:

$$\text{Conc. of Sample} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times \text{Conc. of Standard}$$

Inorganic Phosphorus Assay:

Principle: Phosphorus reacts with ammonium molybdate in the presence of sulphuric acid to form phosphomolybdate complex which is measured at 340nm

Procedure

Reagent blank	Standard	Sample
Sample	-	- 10µl
Standard	-	10µl -
Distilled water	10µl	- -
Working Reagent	1ml	1ml1ml

Mix and incubate at room temperature for 10mins or 5mins at 37^0C, measure the absorbance of the standard and sample against the reagent blank at 340nm.

CALCULATION:

$$\text{Conc. of Sample} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times \text{Conc. of Standard}$$

Histological Assessment of Tissue

The animals were sacrificed periodically and dissected to obtain the heart, liver and kidney for histological studies. The tissue samples were immediately preserved by immersion into 10% formalin. The fixed tissue samples were cleared in xylene and embedded in paraffin wax and sections were cut using 5-micron in a rotatory microtome. The sections were then examined using the light microscopy after staining with hematoxylin and eosin for general tissue structures. The photomicrograph were then finally interpreted by an expert histologist.

STATISTICAL ANALYSIS: Data was expressed as mean ± standard deviation. The significant difference between the test and control were analysed using unpaired t-test. Data were analysed using the SPSS software (SPSS Inc. Chicago, USA). $P \leq 0.05$ was set at the level of significance.

III. RESULTS

Results are summarized in tables 1 The table 1 below, shows the mean plasma calcium and inorganic phosphate concentrations in normal female albino rats following oral administration of the decoction of lime (*Citrus aurantifolia*) and Lipton tea (*Camellia sinensis*) for 14 days. The result showed that the plasma calcium concentration significantly increased compared to normal control rats ($P<0.05$) with a non-significant change in phosphate levels.Table 2 shows the mean weight of rats. The rats in the control group experienced a continuous fluctuation in body weight (g) throughout the period of the study with a non-significant weight elevation especially on day 14. Histological examination showed relatively normal liver, kidneys and heart in figs 1,2 and 3 respectively.

Table 1: Mean plasmacalcium and inorganic phosphorus concentration (mg/dl) in normal rats after 14 days of administration of lime(*Citrus aurantifolia)* and Lipton tea(*Camellia sinensis)* decoction.

Group/Days	Day 0	Day 1	Day 7	Day 14
Control(Calcium)mg/dl	8.61 ± 0.16^a	10.23 ± 0.48^a	13.12 ± 0.67^a	14.46 ± 0.97^a
Test (Calcium)	8.99 ± 1.57^a	10.65 ± 0.87^a	14.51 ± 1.78^b	15.92 ± 1.94^b
Control (Phosphate) mg/dl	6.67 ± 1.44^a	5.81 ± 0.01^a	6.75 ± 0.01^a	5.23 ± 0.003^a

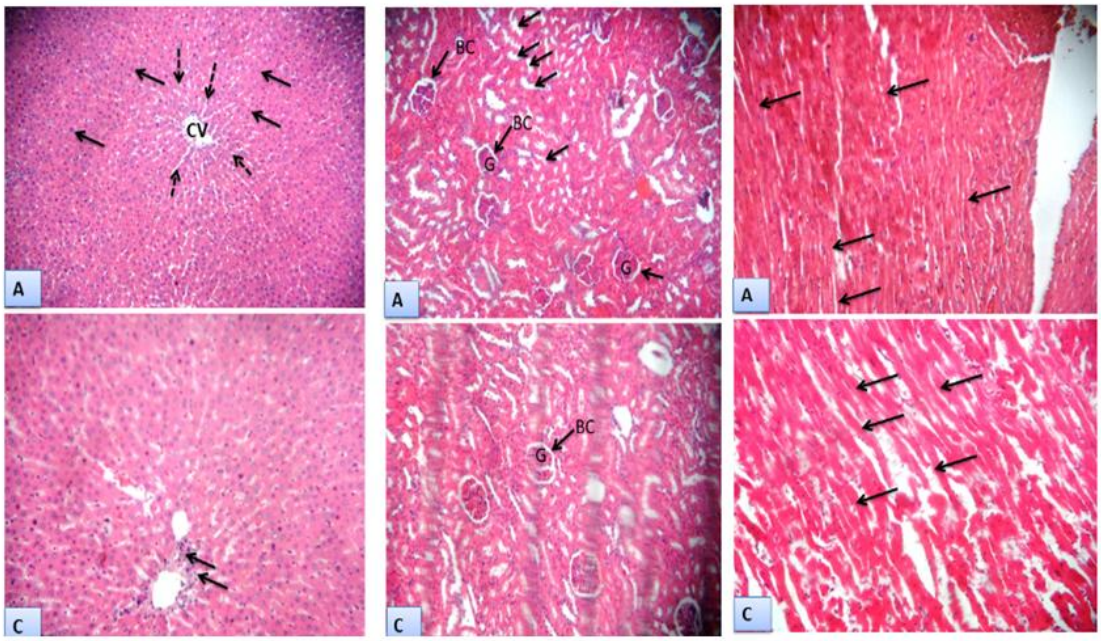
Test (Phosphate)	9.17±2.89[a]	6.52±0.06[a]	6.82±0.02[a]	5.13±0.007[a]

Values are presented as mean±SD of triplicate determinations. Values with different superscript alphabets are statistically significant at (P<0.05).

Table 2: Mean weight(g) of rats administered decoction of *Citrus aurantifolia(*lime) and *Camellia sinensis*(Lipton tea).

Group/Days	Day 0	Day 1	Day 7	Day 14
Control	141.68±4.44[a]	151.03±5.77[a]	144.97±27.18[a]	171.78±21.23[a]
Test	130.417±8.49[a]	150.28±5.95[a]	156.48±11.21[a]	159.02±34.73[a]

Values are presented as mean±SD of triplicate determinations. Values are statistically significant at p<0.05.



Histopathology of Liver, Kidney and Heart in figs 1,2 and 3 respectively

Figure 1: (Photomicrograph of Liver): Group A (Control) shows normal Central vein= **CV**, with well radiating Hepatocytes (**Arrow**) and sinusoids (**Dash arrow)**. Group C has normal hepatocytes and stroma but are mildly infiltrated around the portal triad (**Arrow**)

Figure 2: Photomicrograph of Kidney: Group A (Normal control) with normal Glomeruli (**G**), Bowman's capsule (**BC**) and Renal Tubule (**RT**). **Group** C is similar to the control.

Figure 3: Photomicrograph of the Heart: Group A (control) with parallel pattern of myofibrils with centrally located nuclei (**Arrow**), alongside their branches. Groups C is similar to the control (Group **A**). **H&E x100**

IV. DISCUSSION

Medicinal plants are the nature's gift to human beings to help them pursue a disease-free healthy life. Plants are a valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavours, fragrances, colours, biopesticides and food additives.[18]The previous pharmacological studies revealed that citrus fruits and the leaves of camellia sinensis used in the preparation of tea possessed antimicrobial, anthelmintic, insect repellent, antioxidant, anticancer, cardiovascular, central nervous, antiinflammatory, analgesic, antidiabetic, reproductive, gastrointestinal, immunological, respiratory and many other pharmacological effects.[17]

The result of this study showed significant elevation of plasma calcium levels with combined decoction of *Citrus aurantifolia* and *Camellia sinensis*. This is in keeping with Nagwa et al[9] stating the protective effects of *Citrus sinensis* and *Citrus aurantifolia* against osteoporosis and their phytochemical constituents in ovariectomised rat model. This also corroborates with study by Sohair et al[19] reporting the possible anti-osteoporotic mechanism of *Cicer arietinum* extract in ovariectomised rat and suppressed osteoclastogenesis in vitro as reported by Liu et al.[20] The probable mechanism of preventing bone loss was by inhibition of NF-kB ligand (RANKL) induced osteoclastogenesis, decreasing osteoclast number, preventing bone loss and restoring bone strength in mice. In yet another study,[15] by Das et al ovariectomized rats prone to osteoporosis were supplemented with black tea extracts which reported improved calcium and phosphate levels with

subsequent improvement in bone density. It was therefore, suggested that aqueous black tea extract may be effective in preventing bone loss due to ovarian hormone deficiency. This could also explain the phytoestrogenic effects of black tea extract in an oophorectomised rat model of osteoporosis and the anti osteoporotic effects of green tea extract in rats intoxicated carbon tetrachloride as reported in previous studies.[21,22] The mechanism of these extracts in preventing osteoporosis is not clearly understood, hence further studies are needed in this direction

From the foregoing, it is evident that either lime or tea has some anti-osteoporotic effects. Therefore the combined decoction of both has additive effect which further enhanced anti-osteoporotic effect in the female rats studied. This could have promising effects in preventing osteoporosis especially in ovariectomized rats as reported in earlier studies and by extension in female humans with premenopausal symptoms and in the menopausal age groups.

V. CONCLUSION:

In conclusion, the present study showed that the combination of tea and limedecoction increased the plasma calcium concentration, which in turn would physiologically bring about increased trabecular bone mineral content and bone mineral density of tibia and have anti-osteoporotic effect. The mechanism of action is not clearly known, therefore, further work is suggested to understand their cause and effects in order to aid their popularity of use as medicinal plants, drug supplements and adjunct therapy for bone disorders especially in preventing osteoporosis in vulnerable females.

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