

Original Research

Evaluation of *Citrus sinensis* (Osbeck) (Rutaceae) Peel Preparation on The Reduction of Creatinine Levels and Its Antioxidant Properties In-Vitro.

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ABSTRACT

Background: The incidence of kidney diseases is on the increase globally, which necessitate the need for a safer alternative that will mitigate the effect of kidney disease. The study assessed the ability of *Citrus sinensis* peel extracts to reduce creatinine, a significant biomarker for glomerular filtration rate, a test that measures overall efficiency of the kidney.

Method: *Citrus sinensis* peels were collected, identified and authenticated by a curator. Phytochemical screening was carried out using standard procedures. Antioxidant activity was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay compared to Vitamin E standard. All experiments were carried out in triplicates. Two-way analysis of variance (ANOVA) was used to compare the difference between the antioxidant activities of extracts and standard drugs at 95% confidence. Amount of creatinine was determined using the Jaffe method before and after the addition of the extract to determine the effect of the extract on the amount of creatinine in the urine samples. The creatinine calculated to be present was analysed statistically using STATA MP 16.

Results: Extract was rich in saponins, terpenoids, tannins, flavonoids, reducing sugars and unsaturated lactone. The fresh peel extract at a concentration of 1% exhibited a DPPH antioxidant activity of $89.44\% \pm 0.3$,

and the dried peel extract at a concentration of 1% indicated a DPPH antioxidant activity of $79.16\% \pm 1.03$ compared to the Vitamin E standard used. The extract showed statistically inferior antioxidant activities compared to the standard drug across all concentrations ($p < 0.05$). The significant Creatinine reduction observed for the fresh peel extract was 0.001, 0.005 and 0.173 for the morning, afternoon and evening urine samples, respectively. The significant Creatinine reduction observed for the dried peel extract was 0.008, 0.034 and 0.085 for the morning afternoon and evening urine samples, respectively. Insignificant differences of 0.604, 0.626 and 0.331 were gotten upon comparison of the two extracts which indicated that both the extracts are equally as effective in Creatinine level reduction.

Conclusion: From the study carried out, the extracts were rich in secondary metabolites, the extracts had suitable antiradical scavenging property on DPPH when compared with Vitamin E, and the extracts had, to a certain degree the ability to reduce creatinine levels.

INTRODUCTION

The kidneys maintain the blood creatinine in a normal range. Creatinine is a relatively reliable indicator of kidney function. Elevated creatinine level signifies impaired kidney function or kidney disease¹. Whenever the kidneys become damaged for any reason, the creatinine level in the blood will rise due to the low clearance of creatinine by the kidneys. Abnormally high levels of creatinine thus warn of possible malfunction or failure of the kidneys. It is for this reason that standard blood tests routinely check the amount of creatinine in the blood. A more precise measure of the kidney function can be estimated by calculating how much creatinine is cleared from the body by the kidneys. This measure is referred to as creatinine clearance, and it estimates the rate of filtration by kidneys (Glomerular Filtration Rate GFR). Average creatinine clearance for healthy women is 88-128 mL/min and 97 to 137 mL/min in males (normal levels may vary slightly between labs)². Irrespective of its cause, kidney disease is associated with a decrease in GFR, and the severity of kidney disease correlates closely but inversely with GFR. A normal GFR (~125 mL/min) is presumptive evidence of healthy, functioning kidneys. As GFR (i.e. kidney function) declines, urinary excretion of creatinine also declines and blood concentration of creatinine increases. Although reduced GFR (i.e. renal disease) is also associated with increased

plasma urea concentration, other non-renal conditions can give rise to increased plasma urea. Interpretation of increased plasma urea is often aided by simultaneous measurement of creatinine and calculation of the urea: creatinine ratio to establish a renal or non-renal cause². The most commonly used endogenous marker for the assessment of Glomerular Filtration Rate (GFR) is creatinine. The calculated clearance of creatinine is used to provide an indicator of GFR. This involves collecting urine over 24 hours or preferably over an accurately timed period of 5 to 8 hours since 24-hour collections are notoriously unreliable. Creatinine clearance is then calculated using the equation:

$$C = (U \times V) / P \dots\dots\dots \text{Equation 1}^3$$

C = clearance,
U = urinary concentration
V = urinary flow rate (volume/time i.e. mL/min),
and
P = plasma concentration.

Creatinine clearance should be corrected for body surface area. Improper or incomplete urine collection is one of the significant issues affecting the accuracy of this test; hence timed collection is advantageous. Furthermore, due to tubular secretion, creatinine overestimates GFR by around 10% to 20%. GFR is classified into the following stages based on the Kidney Disease Improving Global Outcomes (KDIGO) stages of chronic kidney disease (CKD):

- Stage 1 GFR greater than

90 mL/min/1.73 m

- Stage 2 GFR-between 60 to 89 mL/min/1.73 m
- Stage 3a GFR 45 to 59 mL/min/1.73 m
- Stage 3b GFR 30 to 44 mL/min/1.73 m
- Stage 4 GFR of 15 to 29 mL/min/1.73 m
- Stage 5-GFR less than 15 mL/min/1.73 m (end-stage renal disease)

Urine analysis involves an assessment of urine characteristics to aid in disease diagnosis and consists of physical observation, chemical, and microscopic examination⁴. Physical observation involves assessing colour and clarity. The normal colour of urine is straw-coloured in the presence of dehydration urine is a darker colour. Red urine may indicate haematuria or porphyria or represent the dietary intake of food like beets. Cloudy urine may be seen in the presence of pyuria due to urinary tract infection. Specific gravity is an indicator of renal concentrating ability that may be measured using refractometry or chemically using urine dipstick. The physiologic range for specific gravity is 1.003 to 1.030 and is increased with concentrated urine and decreased with dilute urine⁵. The kidney undergoes irreversible and progressive deterioration, and the resultant high cost of treatment is not readily available for individuals, families and government at large. Kidney damage is a significant determinant for the development and progression of accelerated atherosclerosis, ischemic vascular

disease, and cardiovascular events. Individuals with even the earliest signs of CKD are at increased risk of cardiovascular disease and may die long before they reach end-stage renal disease (ESRD)⁶.

The study aims to investigate the effect of *Citrus sinensis* preparations on the reduction of creatinine level and its antioxidant activity in vitro. *Citrus X sinensis* (L.) Osbeck (Rutaceae) is commonly called Orange, Sweet orange. It thrives in subtropical areas where the temperature usually doesn't drop below freezing, and there is a moderate amount of rainfall.

Optimal growing temperature falls around 550 to 1000F, and orange trees are susceptible to being killed off by cold temperatures, especially with younger trees. Soil needs to be deep enough to allow the tree's roots to develop properly to ensure the proper nutrient exchange and anchor the tree⁷.

Medicinally, orange and orange juice increases iron absorption from foods; this has been shown to reduce anaemia. Oranges are also a good source of absorbable folic acid⁸.

Honey is the saccharine liquid prepared from the nectar of the flowers by the hive-bee *Apis mellifica* and bees of other species of *Apis*. It is produced in certain parts of the West Indies, California, Chile, Africa, Australia, New Zealand and India. Honey is viscid, translucent, and white to pale yellow or yellow-brown- coloured liquid, on keeping it, crystals of glucose separate. Odour is pleasant and characteristic, and the taste is sweet.

The odour and taste depend on the flowers from which the nectar is sucked. The specific rotation of honey is +3° to -10° and total ash 0.1 to 0.8%.

Honey represents a natural product that does not carry side effects, unlike synthetic drugs which can be harmful to health. Among the compounds found in honey are vitamin C, phenol compounds, catalase, peroxides, glucose oxidase enzymes have antioxidant properties⁹. Various polyphenols are reported in honey.

METHOD

Reagents/Solutions Used: These reagents and chemicals were used: 10% Naphthol, Concentrated Sulphuric acid (BDH Binder, Germany), Fehling's Solution A (Sigma Aldrich Germany), Fehling's Solution B (Sigma Aldrich Germany), 2% Sulphuric acid (BDH Binder, Germany), Mayer's reagent (CDH Fine Chemical), Chloroform, Dragendorff's reagent (Merck), Dilute Hydrochloric acid (BDH Binder, Germany), Ferric chloride solution (OEM manufacturers India), Sodium Hydroxide, Glacial acetic acid (GFS Chemicals, Inc., Columbus), Water.

Apparatus Used: Hot air oven, separation funnels (Biomate 3, USA), chemical balance, water bath, volumetric flask, test tubes, test tube racks, measuring cylinder, beakers, evaporating dishes, muslin cloth, pipettes, cotton wool, volumetric flask.

Collection and Preparation of

Plant Material: Sweet orange peels were collected and authenticated by the laboratory staff on the 12th of

July 2019. The collection was done for one week around Idi-Araba environs in Lagos state. A portion was dried in the oven at 50°C for 24 hours, and the other portion was left to prepare the fresh peel extract. After proper drying of the peels, it was then pulverised using the mechanical grinder for adequate size reduction.

Extraction from Orange Peels: A total of 1.7kg of the pulverised orange peel was weighed and transferred into a plastic bowl, and 6.25L of water was added. Next, 0.5L of fresh, natural honey was added to the container. The contents of the container were carefully stirred until it was all properly mixed. It was boiled for about 15 minutes and then stirred once again. The dried peel preparation was then covered with black polythene nylon to aid the fermentation process.

The same process was done with fresh peels. 1.7kg of fresh peels was weighed and transferred into a plastic bowl, and 6.25L of water was added. 0.5L of fresh, natural honey was added to the container. The contents were stirred carefully to get a proper mix. It was boiled for about 15 minutes and stirred again. The container was covered with black polythene nylon to aid the process.

Both containers were labelled correctly and kept in the dark for 28 days (4 weeks) for the fermentation process to occur.

Phytochemical Screening:

Orange peel preparation was investigated for phytochemicals using standard procedures¹⁰. Test for Saponins, Alkaloids

Triterpenoids, Tannins, Shinoda, Fehling's, Keller killiani, Anthraquinone, Kedde, Molisch, and Shinoda's Tests were carried out.

Test for Honey

The honey used for the fermentation process was tested using the following method: Fieche's test: 3ml of honey was mixed with 2ml of ether thoroughly, the two layers obtained was separated and evaporated to dryness. The upper ethereal layer was put in a petri dish and evaporated. 1% resorcinol and HCl was added to the residue.

Molisch's Test: Honey is treated with alpha Naphthol and concentrated sulphuric acid

Reducing Sugar Test: A little amount of the honey was heated with drops of a mixture of Fehling's solution A & B Flocculation test and solubility test: Solubility test in water and alcohol was also carried out on the honey sample both in cold and boiling solvent.

Determination of Creatinine in

Urine: 0.25ml of urine sample was taken from a healthy subject into 25ml stoppered cylinder, which was made up with distilled water (1:100 dilution factor). 3ml was taken from the diluted sample into a test tube, and 1ml of 0.04N picric acid was added, followed by 1ml of 0.5N NaOH. The test tube was shaken and left to stand for 20 minutes, there was a colour change, and the intensity of colour change was determined in the UV

spectrophotometer at 500nm. This procedure was repeated for all urine samples collected, and readings were taken in triplicates.

Determination of The Effect of Extracts on Creatinine in Urine Samples:

Five different concentrations of the extracts were prepared (10%, 20%, 30%, 40%, 50%). 0.25ml of the urine sample was pipetted into a stoppered cylinder; the solution was made up to mark with distilled water to obtain a 1:100 dilution factor. 3ml of the diluted urine sample was pipetted into a test tube, 1ml of each of the extract was added into different test tubes. The content was shaken and left to stand for 20 minutes. There was a colour change in the mixture, and the intensity of the colour was determined using UV spectrometer at a wavelength of 500nm.

The procedure was repeated for all the urine samples collected at different concentrations of the extracts and readings were taken in triplicate.

Determination of DPPH Free Radical Scavenging Activity:

The antioxidant activity of the extracts was evaluated on the basis of the radical scavenging effect of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-free radical activity by a slightly modified method employed by Blois¹¹. The extracts (0.0125-0.2%) were prepared from the stock solution using suitable dilution. 0.1mM of DPPH was prepared in

methanol, and 1 mL of this solution was mixed with 3 ml of the sample solution in test tubes in triplicates. These solution mixtures were shaken vigorously, then were incubated for 30 min and their absorbances were measured at 517 nm using UV-VIS Spectrophotometer. Methanol (3 mL) with DPPH solution (0.1mM, 1 mL) was used as control. Methanol was used as blank. The % inhibition was calculated by the formula given below:

$$\text{Inhibition of DPPH} = \frac{(A_c - A_a)}{A_c} \times 100\% \dots \dots \dots \text{Equation 2}$$

Where A_c is the absorption of the control sample, and A_a is the absorption of the tested extract¹².

RESULTS

Phytochemical Screening

The table below shows the results of the phytochemical screening of the fresh and dried peel extract of *Citrus sinensis*.

PHYTOCHEMICAL SCREENING

The table below shows the results of the phytochemical screening of the fresh and dried peel extract of *Citrus sinensis*.

Table 1: Phytochemical Screening

Phytochemical test	Fresh peel preparation	Dry peel preparation
Saponins	–	–
Alkaloids	–	–
Triterpenoids	–	–
Tannins	+	+
Shinoda	+	+
Fehling's	+	+
Keller killiani	+	+
Anthraquinone	+	–
Kedde	+	+
Molisch	+	+
Shinoda's Test	+	+

Honey test

The table below shows the results of the phytochemical screening of honey used in the fermentation process

Table 2: Honey test

Test	Honey reaction
Solubility in absolute ethanol	Soluble
Solubility in water	Insoluble
Flocculation test	Gum/mucilage absent
Reducing sugar test	Present
Fieche's test	Transient pink obtained. Honey is pure
Molisch's Test	Positive test

ANTIOXIDANT TESTING

Table 3 shows the results Percentage Inhibition of Fresh and Dried Orange Peel on DPPH Antiradical Assay

Table 3: Percentage Inhibition of Fresh and Dried Orange Peel from DPPH Antiradical Assay

Concentration	1			2			3			4			5		
(%)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Samples															
Vitamin E	50.14	58.67	49.14	93.04	93.64	93.71	93.62	93.35	92.00	93.62	93.93	92.57	92.46	93.35	92.86
Fresh peel	89.86	89.60	88.86	88.12	88.44	88.86	86.96	86.99	85.71	84.64	84.97	83.71	83.48	83.24	83.71
Dried peel	81.16	78.61	77.71	76.52	72.54	71.43	71.88	65.61	64.29	57.68	56.07	55.43	53.04	52.31	54.57

Statistical Result from DPPH Antiradical Activity

Table 4 gives the statistical analysis of the fresh and dried peel fermented product against Vitamin E standard, while figure 1 is the diagrammatical representation.

Table 4: Statistical Result from DPPH Antiradical Activity Assay

Percentage	1	2	3	4	5
Sample					
Vitamin E	52.65 ± 3.02	93.47 ± 0.21	92.99 ± 0.50	93.38 ± 0.41	92.89 ± 0.26
Fresh Peel	89.44 ± 0.30 *	88.47 ± 0.21 ****	86.56 ± 0.42 ****	84.44 ± 0.38 ****	83.48 ± 0.14 **
Dried Peel	79.16 ± 1.03 *	73.5 ± 1.55 *	67.26 ± 2.34 *	56.39 ± 0.67 ****	53.31 ± 0.67 ****

* = Significantly different from Vitamin E at $p \leq 0.05$ ($p \leq 0.01$)

** = Significantly different from Vitamin E at $p \leq 0.05$ ($p \leq 0.005$)

*** = Significantly different from Vitamin E at $p \leq 0.05$ ($p \leq 0.001$)

**** = Significantly different from Vitamin E at $p \leq 0.05$ ($p \leq 0.0001$)

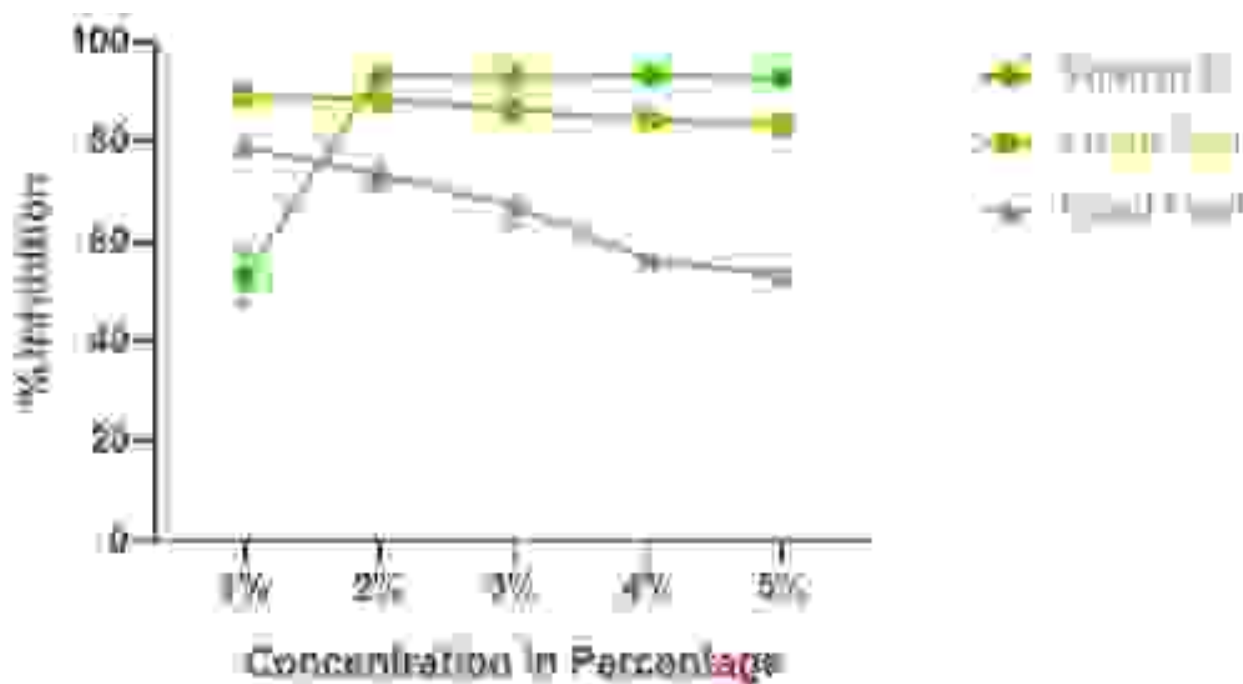


Fig 1: Graph Of % Inhibition of DPPH Against % Concentration of Test Substance

Creatinine inhibitory activities of the extract

The amount of creatinine present in the urine samples was determined before and after the addition of the extract. Table 5 shows Concentration of Creatinine in Urine Sample Without Extract while table 6 shows the Concentration of Creatinine in Urine Sample with the fresh peel extract and table 7 shows the Concentration of Creatinine in Urine Sample with the dried peel extract

Table 5: Concentration of creatinine in urine sample without extract

AMOUNT OF CREATININE IN URINE SAMPLES WITHOUT EXTRACTS (g/L)			
S/N	6AM	12PM	6PM
DAY 1	44.2	47.94	52.54
DAY 2	50.25	50.81	50.25
DAY 3	51.19	47.25	40.51
DAY 4	51.16	50.82	49.58

The amount of Creatinine present was calculated using the following formula:

$$\text{mg creatinine} \frac{2000}{200\text{cm}^3} = (\text{Absorbance of test} \div \text{Absorbance of standard}) \times 0.015 \times \frac{2000}{3}$$

.....Equation 3

Table 6: Concentration of Creatinine in Urine Sample with Fresh Peel Extract

AMOUNT OF CREATINE IN URINE SAMPLES WITH FRESH PEEL EXTRACT (g/L)				
DAY	CONCENTRATION	6AM	12PM	6PM
DAY 1	10%	21.13	22.13	20.89
	20%	22.24	17.32	21.01
	30%	23.53	26.12	25.4
	40%	31.21	33.31	35.95
	50%	37.13	41.03	42.35
DAY 2	10%	18.87	26.47	22.52
	20%	24.61	25.52	28.84
	30%	34.56	38.21	37.54
	40%	46.47	43.01	42.74
	50%	44.4	47.21	45.23
DAY 3	10%	22.76	21.77	24.08
	20%	33.24	32.68	32.56
	30%	37.09	36.87	36.75
	40%	38.55	38.17	40.62
	50%	43.86	47.27	48.02
DAY 4	10%	17.51	23.55	20.85
	20%	27.71	22.32	24.81
	30%	30.49	29.67	31.81
	40%	36.68	35.62	35.88
	50%	44.37	46.54	41.94

Table 7: Concentration of Creatinine in Urine Samples with Dried Peel Extract

AMOUNT OF CREATINE IN URINE SAMPLES WITH DRIED PEEL EXTRACT (g/L)				
DAY	CONC ENTRATION	6AM	12PM	6PM
DAY 1	10%	20.93	18.43	22.23
	20%	23.56	25.27	27.78
	30%	31.56	37.05	37.89
	40%	39.71	40.81	40.92
	50%	41.21	42.34	42.76
DAY 2	10%	22.21	22.65	22.67
	20%	30.33	30.34	29.49
	30%	35.71	37.05	38.06
	40%	37.55	39.27	39.75
	50%	40.02	41.13	41.81
DAY 3	10%	20.06	21.42	22.21
	20%	28.31	27.19	28.72
	30%	36.41	34.68	34.15
	40%	36.72	37.64	40.21
	50%	40.12	42.21	43.14
DAY 4	10%	21.97	23.05	22.02
	20%	25.24	26.41	23.42
	30%	34.91	37.75	38.51
	40%	40.12	39.98	40.78
	50%	40.75	41.74	42.47

DISCUSSION

The incidence and prevalence of CKD are thought to be on the increase globally⁶. Sub-Saharan Africa bears more of the burden appears to be more marked because CKD tends to affect relatively younger individuals, most of which are in the economically productive age group. Creatinine, non-protein nitrogen (NPN) waste product, is produced from the breakdown of creatine and phosphocreatine and can also serve as an indicator of renal function. Creatine is synthesised in the liver, pancreas, and kidneys from the transamination of the amino acids, e.g. arginine, glycine, and methionine¹³. Creatine then circulates throughout the body and is converted to phosphocreatine by the process of phosphorylation in the skeletal muscle and brain. The majority of the creatinine is produced in the muscle. As a result, the concentration of plasma creatinine is influenced by the patient's muscle mass. Compared to BUN, creatinine is less affected by diet and more suitable as an indicator of renal function¹³. Among kidney researchers, Jaffe is well known for having given his name to an analytical principle for assaying creatinine in human body fluids. In 1886, Jaffe observed that a red colour formed when creatinine reacted with picric acid in an alkaline medium. In his landmark paper, Jaffe discussed that the alkaline picrate reaction could also be observed to a much lesser extent with several organic compounds (e.g. acetone, glucose)¹⁴. Jaffe

reaction is a colourimetric method used in clinical chemistry to determine creatinine levels in blood and urine; the colour change that occurs is directly proportional to the concentration of creatinine. The intensity of the colour change was measured at 500nm with an ultraviolet spectrophotometer and recorded¹⁵.

The extraction method employed in this study is via fermentation with honey. The fermentation process of obtaining extracts uses bacteria present in the atmosphere to interact with the sample and carry out the extraction as opposed to extraction with chemical reagents. This method is slower when compared with extraction with chemical reagents as this took 28 days but is a considerable alternative as a natural process. The preliminary qualitative phytochemical analysis using the aforementioned tests and a few others revealed the presence of reducing sugars, cardiac glycosides, phenolic compounds, tannins in both of the preparations and the presence of Anthraquinones only in Fresh peel preparation. These secondary metabolites have many biological and therapeutic properties, and this indicates that the fresh and dried *Citrus sinensis* peel preparations have potential medicinal properties as the preparations contained a good number of these secondary metabolites upon preliminary qualitative phytochemical analysis. From the DPPH antiradical activity assay carried out on the extracts, it was observed that the Fresh peel preparation was statistically inferior

to Vitamin E (standard) at 1%, 2%, 3%, 4% and 5%. Also, the Dried peel preparation was also observed to be statistically inferior to the Vitamin E standard at 1%, 2%, 3%, 4% and 5%. The highest inhibition for the Fresh peel extract was observed to be $89.44\% \pm 0.30$, and that of Dried Peel was observed to be $79.16\% \pm 1.03$ compared to Vitamin E. The above values indicate that the fresh peel extract had higher antioxidant properties when compared to that of the dried peel extract.

The high antioxidant activity of *Citrus sinensis* peels should be attributed to the presence of various classes of flavonoids and other phenolic contents. On comparison with the control, i.e. urine sample without *Citrus sinensis* preparations, the Fresh peel preparation showed a for the morning, afternoon and evening samples ($p < 0.05$). This infers that there was a degree of considerable inhibition of creatinine by the fresh peel *Citrus sinensis* preparation. Also, on comparison of the dried peel preparation with the control, the dried peel *Citrus sinensis* preparation showed a significant difference for the morning, afternoon and evening samples ($p < 0.05$). This infers that there was a degree of substantial inhibition of creatinine levels by the dried *Citrus sinensis* preparation. The dried and fresh peel preparations were also compared against each other to determine which is more effective, but there was no significant difference. This shows that both the fresh and dried preparations of *Citrus sinensis* peels are equally as

effective in the reduction of creatinine levels.

CONCLUSION

This study was able to confirm the presence of some biologically and therapeutically important compounds such as reducing sugars, cardiac glycosides, phenolic compounds, tannins from the orange peel preparation. This shows the potential ability of the extracts to be useful in managing, control or even prevention of various diseases. *Citrus X sinensis* preparations gave significant antioxidant property and lowered creatinine level in the urine sample used. It infers that the preparation can be used to mitigate the effect of kidney damage, thus slowing the progression of kidney damage from stage one to stage five of kidney diseases. *Citrus X sinensis* peel preparations will be useful in combating oxidative stress which is one of the predisposing factors to neurodegenerative diseases and in the management of various kidney diseases.

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