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Comparing Day 0 and Day 21, Hematological and Biochemical Changes on Male White *Rattus norvegicus* Exposed to *Carissa edulis*, Water and Ethanolic Extracts, a Herb Commonly used in Management of Diabetes Mellitus in Baringo, County Kenya

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Abstract

The use of Carissa edulis in the management of Diabetes Mellitus in Baringo County and other parts of Kenya is steadily increasing. This is so, despite the fact that there is a knowledge gap on the Hemato-biochemical responses, on those exposed to it. To investigate the hematological and biochemical changes/responses of Carissa edulis extracts (ethanolic and aqueous) fed to white Rattus norvegicus once daily via oral gavage for 21 days. The study employed an experimental study design. Two kilograms (2 Kg.) of Carissa edulis (CE) roots & back mixtures, were collected from Baringo County, Kenya, where they are commonly used by herbalists in the treatment of diabetes mellitus. They were cleaned at the place of origin, labeled, and transported to the University of Eldoret, Kenya, where its identity was confirmed using their morphological characteristics. Ethanolic and aqueous extraction was done, yield amounts determined and qualitative screening of phytochemicals done. Fifteen (15) male white Rattus norvegicus rats, obtained from the University of Eldoret, Department of biological Sciences were randomly chosen, and caged in 3 cages of 5 rats each. This is after seven (7) days acclimatization, (making the rats adapt the laboratory environment). Sick and rats with abnormalities were excluded in the experiment. Cage 1, 2 and 3, were given orally, ethanolic CE extracts, aqueous CE extracts, and distilled water (controls), respectively. These extracts were given once every day in the morning for 21 days. Blood samples for hematological, (hemoglobin [Hb], red blood cells [RBC], Lymphocytes (LYMPH), Monocytes (MONO), Hematocrit [HCT], Mean Corpuscular Volume [MCV] Mean Corpuscular Hemoglobin [MCH], Mean Corpuscular Hemoglobin Concentration [MCHC] and white blood cells [WBC]) and, biochemical (Urea, creatinine, Alanine Transaminase [ALTs], Aspartate Aminotransferase [ASTs]) tests, were drawn from the rats' tail end at days 0 and 21. Blood analyzed compared and later the findings presented in figures and in a tabular form. Analysis of the blood was done using using ADVIA 220i for hematology and Chemistry analyzer Cobas C311 for biochemicals. Ethical approval to conduct this study was granted by Animal Research and Ethics Committee (HAREC) of University of Eastern Africa Baraton (UEAB) - Kenya (REC: UEAB/3/1/2018). There was a slight increase in RBC counts, with reference to rats exposed to ethanolic extracted extracts and so to the control groups, but a slight decrease in those exposed to water extracted extracts. Hemoglobin values in both the

groups increased (rats exposed to water extracted extracts and rats exposed to ethanolic extracted extracts) but a slight decrease was seen in control groups. With reference to WBC counts, treated animals witnessed a decrease while controls saw an increase. All the tested hematological changes were within the normal ranges. Regarding biochemical parameters tested, in all the rats exposed to the water and ethanolic extracted extracts, their urea levels showed an increase at day 21, while a mixed result was seen in the creatinine parameter – an increase in controls, but a decrease in those exposed to ethanolic extracted extracts and no change was seen those exposed to water extracted extracts. Controls recorded a slight but insignificant drop in ALTs, but an increase in both water and ethanolic extracted extracts. Concerning ASTs, there was a slight increase in controls, a scenario witnessed in both water and ethanolic extracted extracts. The overall changes in all the tested biochemical parameters were also within the normal ranges. Carissa edulis aqueous and ethanolic extracts, given orally to male Wistar albino rats, did not induce any significant interference to the hematological and biochemical parameters, though much more studies in a large scale (use of more rats), at different doses are needed.

Keywords: *Carissa edulis,* Ethanolic & Aqueous extracts, Hematological & Biochemical P arameters changes, day 0 and day 21, white *Rattus norvegicus*

INTRODUCTION

Currently, the principal use of herbal medicines for health promotion and therapy for chronic and life-threatening conditions is increasing. And this could be because of the belief that conventional treatment is ineffective (Klemow et al., 2011). Hence the increasing concerns for herbal treatments' safety (Wachtel-Galor & Benzie, 2011).

In the tests of herbal toxicity, the primary aim is to identify their potential adverse effects and, secondly, their limits of exposure (Ifeoma & Oluwakanyinsola, 2013). The aim being, to reduce or eliminate the risks that may accrue when exposed to human beings. Animals have been used as sentinels for early detection of the potential risks to humans or as models to study the causes, pathogenesis, progression, and treatment of diseases (Iannaccone & Jacob, 2009).

Several parameters are measured to understand the extent of the impacts; physical, histological, hematological, and biochemical parameters, and even mortality. In hematological parameters, one usually examines whether a deviation from the normal, acceptable ranges has occurred or not [n/b, *there are usually acceptable normal ranges of hematological and biochemical parameters*] *(Arika et al., 2016; Giknis & Clifford, 2008; Teixeira et al., 2000)*.

Some of the changes, for instance, low hemoglobin content, indicate iron deficiency anemia (Ogawa et al., 2020; WHO, 2011). High percentage of hematocrit (above 55%) may indicate polycythemia. A high WBC count indicates worsening insulin sensitivity and predicts the development of Type 2 Diabetes (Vozarova et al., 2002). High ALT values, predict liver diseases(Gellert-Kristensen et al., 2020), and high ASTs may signify, the risk of type 2 diabetes(Kunutsor et al., 2014; Meltzer & Everhart, 1997), and deranged urea and creatinine values may indicate deterioration in the kidneys. These changes can be induced by herbs like *Carissa edulis*, a herb commonly used as a medicinal plant in many parts of Kenya, Nigeria, and other parts of the world to manage diabetes, epilepsy, and abdominal ailments(Chebor et al., 2020; Ibrahim et al., 2015).

The tests for toxicity can be classified as either acute, (less than 14 days of exposure), sub-chronic, (between 15 and 28 days) or chronic (above 28 days), (Bello et al., 2016), in relation to the duration. This study, therefore, examined hematological and biochemical impacts of *Carissa edulis*, aqueous and ethanolic extracted extracts exposed to *white Rattus norvegicus* at a determined dosage via oral gavage for 21 days (**sub-chronic)**.

MATERIALS AND METHODS

Study Area

The study was carried out in the University of Eldoret, Biotechnology Centre laboratory.

Study Design: Experimental

Collection, Identification, Preparation and Extraction of the Herb

Collection: Plant materials were collected from Katimok forest- Baringo County, Kenya.

Identification: The herb was identified using morphological characteristics (Beattie et al., 2005) by the University of Eldoret's (Kenya) Department of Biological Sciences, taxonomists.

Preparation: Two Kilograms (2 Kg) of the fresh roots & bark mixtures of CE, were sampled and labeled in place of origin, cleaned with distilled water to remove external debris attached to them, then transported to the University of Eldoret-Kenya, Biotechnology Laboratory, where they were air-dried at room temperature to complete dryness, before being crushed with a grinder- OHMS OCG-200, into a powder form in readiness for extraction.

Extraction

Choice of solvent: The choice of extraction solvents used, were informed by what the herbalists are using on the ground (water) and, in the university lab environment- a solvent safe and closer in polarity to water was chosen (ethanol). The ethanol used, was sourced from *Sigma-Aldrich-Kenya.* Concentration-*98% analytical reagent.* For aqueous extraction, distilled water was used.

Ethanol extraction: Five hundred grams (500 g) of the herbs were soaked in 2 liters of ethanol for 72 hours at room temperature (Azwanida, 2015), after which the resultant mixtures were filtered using Whatman filter paper (No.1) and the filtrate concentrated to dryness using a vacuum-rotary evaporator machine- BUCHI Rotavapor R-3000 at a temperature range of between 40^0C -50^0C. Fifty milliliters (50mls) of distilled water were then added to the container (volumetric flask) containing the concentrated extract, and then, using a stirring rod, the contents were stirred to dissolve the extract.

Aqueous extraction: Five hundred grams (500g) of the herb was soaked in 2 liters of distilled water at room temperature for 72 hours in an airtight container to minimize mold growth. The resultant mixture was then filtered using Whatman filter paper (No.1), and the filtrate was concentrated to 50 ml using a vacuum-rotary evaporator machine at a temperature between 85^0C-90^0C.

Determination of extraction yield: After extraction, *Carissa edulis*, percentage (%) yield was determined based on the formula described by (Qaid, 2020). The extracts (ethanol and water extract) were lyophilized to complete dryness with the help of '*Harvest Right freeze drier (U.S.A, 2000)'*.

For the ethanolic and aqueous extracts, the resultant yield powder were then, dissolved in 200mls of distilled water. The feeding protocol of the rats was done in reference to (Piero et al., 2011).

Determination of phytochemicals: A total of 5mls were used in phytochemical screening. The screening was done as per the description of (Muralidharan, 2015).

Quantification of the extracts: The screening was done using Shimadzu HPLC SYSTEM Machine from shimadzu cooperation Kyoto-japan. The column was: Silica 250 x 4 nm.

Collection, Acclimatization, Feeding of the Rats

Collection; Fifteen (15) male Wistar albino rats (*Rattus norvegicus)*, between the ages of 4 and 6 months, weighing in the ranges of 200-230g, were recruited. The animals were obtained from the University of Eldoret, Department of biological sciences.

Acclimatization: Before the experiment commenced, the rats were acclimatized in the lab environment for seven (7) days, after which they were randomly caged in three cages of 5 rats each. The aim of acclimatization was to make the rats adapt well to the laboratory environment.

Feeding of the rats before the experiment: The rats were cared for under the laboratory procedure by National Research Council, 2010), and feeding was done using pellets from UNGA limited-Eldoret Kenya at an approximate amount of between 5 to 6g/100mg/day of a rat (Klurfeld et al., 2021), and given water *ad-libitum*.

Inclusion and exclusion criteria
Normal health male Wistar albino rats

Exclusion criteria: Sick rats; Rats with abnormalities.

Experimental protocol
Feeding of the rats during the experiment
Cage 1 and 2 rats were given ethanolic and aqueous extracts of *Carissa edulis* via oral gavage, respectively after determination of the dosage. In addition, they were also given distilled water *ad*-libitum and pellets from Unga Kenya limited at an approximate amount of between 5 to 6g/100mg/day. The feeding of the cases/treated animals was done once daily at 0.5mls *Carrissa edulis*/100mgof a rat, orally at 0900 hours. Cage 3, the controls were fed with only distilled water *ad*-libitum and pellets from Unga Kenya limited at an approximate amount of between 5 to 6g/100mg/day

Measuring weights and Sugars
Weights were measured using *goldfield digital scientific weighing (GMS 06)* and sugars were taken using *Accu-check^R active glucometer from Roche industries* every day at 9am in the morning for the 21 days

Collection of the blood samples
Was done at day zero (0) and at day twenty-one (21).

Site
These blood samples were collected by puncturing the tail end of the rats a fishers' scalpel blade and blood collected in a 2mls syringe. The approximate amount obtained from each rat was around 1ml and put on heparinized sample bottles**,** where applicable, in readiness for the tests

Analysis
Blood analysis was analyzed using *ADVIA 220i* for hematology related parameters and *Chemistry analyzer Cobas C311* for biochemical related parameters.

Ethical Approval
Approval of the research was done by the Human and Animal Research and Ethics Committee (HAREC) of the University of Eastern Africa Baraton (UEAB) - Kenya (REC: UEAB/3/1/2018)

Data Analysis
The resultant data were entered into excel sheet office 19 and analyzed using SPSS software version 21. Descriptive and inferential statistics were used to describe the data and summarized in tabular form. All the tests were 2-tailed, and were considered significant at a p=0.05.

RESULTS

Percentage Yield of CE after Extraction

Table 1: Yield Outcomes of Extraction Yields

	CE – *Carissa edulis*	
	Ethanolic extract	***Aqueous/water Extract***
A. Dry quantity before extraction in mg of the herb	500,000mg	500,000mg
B. Extracted dry quantity after lyophilization in mg [*nb, these were 50 mls for ethanolic and aqueous extracts*]	6,914.4	23,643
% yield extracted $=^{B}/_{A}*100$	1.38%	4.73%

Table 2: Amounts per 1 ml of CE of the herb after dissolving it in 200 mls of distilled water

	CE	
	Ethanolic extract	***queous/water Extract***
Amount used to dilute	200 mls	200 mls
Concentration (mg/ml) (*If 200 mls=6914.4 What about 1 ml*)	34.572 mg	118.23 mg

Dosages Given to the Rats

Based on previous studies (Ngulde et al., 2013) on CE ethanolic extracts, lowest effective concentration was determined and given via oral gavage to the rats at a dosage of 0.5 ml/100 g/rat once daily at 900 hrs

= for ethanolic extracts, =***17.3 mg/100 g of a rat once daily for 21 days***

= for aqueous extracts, = ***59.1 mg/100 g of a rat once daily for 21 days***.

Phytochemical Screening

Table 3: Qualitative analysis of phytochemicals from water and ethanol extracts of *Carissa edulis*

	Phytochemicals	Water extract	Ethanol extract
1	*Alkaloids*	++	++
2	*Flavonoids*	+ ++	++
3	*Phenols*	+ ++	++
4	*Saponnins*	+ +	+++
5	*Tannins*	+ +	_
6	*Quinones*	_	_
7	*Oxalates*	_	_
8	*Terpenoids*	+ +	_
9	*Glycosides*	+ ++	+++
10	*Steroids*	+	+
11	*Coumarins*	+ +	+
12	*Sterols and Triterpenes*	+	++
13	*Xanthones*	+ +	_
14	*Catechins*	+ +	_
% detected phytochemicals		*12/14* **86%**	*8/14* **57%**

Fourteen phytochemicals were testes from the plant extract of *Carissa Edulis* water and ethanol. 86% of the tested phytochemical were detected in the water extract while 57% was detected in the ethanol extract. Other than quinones and oxalates, the rest of the tested compounds were present at moderate quantities. See Table 3 above.

High Pressure Liquid Chromatography

Figure 1 shows the HPLC chromatogram of *Carissa edulis* water and ethanol extracts, the first injection of ethanol extract produced two peaks defined peaks and one undefined peak. The injection of water extract produced three defined and one undefined peak. Both water and ethanol extracts produced five peaks.

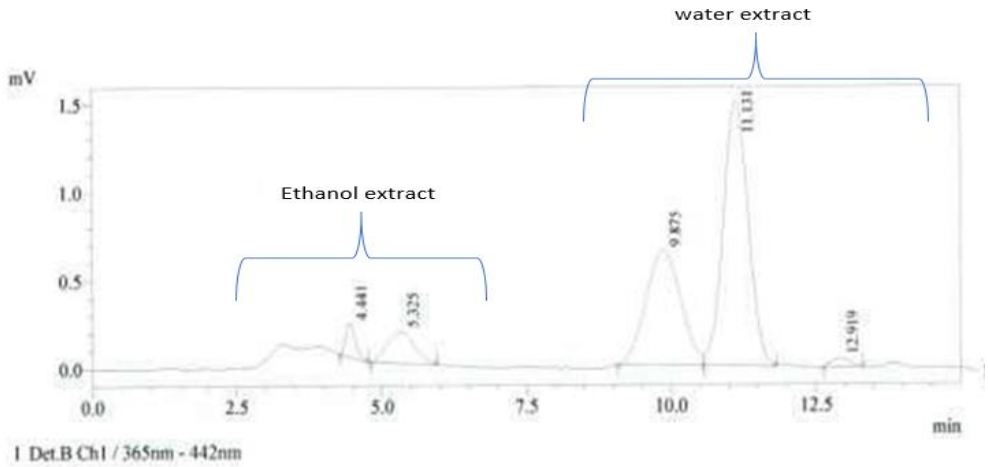

Figure 1: HPLC Chromatogram of water and ethanol extract of Carissa *edulis.*

Weight Averages Changes per Day

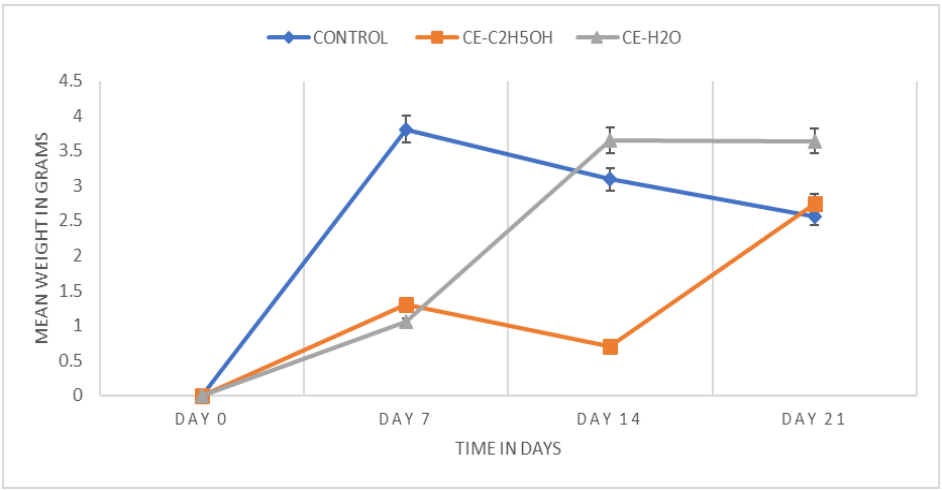

Figure 2: Rate of weight increase/decrease during the 21 days of experiment.

Figure 2 shows the rate of weight increase over the period of the experiment. Control rat's weight increased highly by the mean of 3.81 g per week at the first week. Exposed rats to ethanol and water extracts increased slowly with 1.3 and 1.05, respectively.

Sugar Levels Changes of Rats Exposed to Carissa *edulis*

Table 4 shows levels of sugar from both exposed rats and control rats. The changes witnessed in rats exposed to the ethanolic extracted extracts exposed was on the downward trend, though the decrease was not so much, while in rats exposed water extracted extracts, the changes witnessed was mixed (raised in day 7, decreased in day 14 and raised again in day 21. No significant changes were seen in controls.

Table 4: Mean sugar levels change of rats exposed to both water and ethanol extracts of Carissa *edulis*

Extracts	DAY 0	DAY 7	DAY 14	DAY 21
Controls	12.95±4.8(mmol/L)	11.47±1.2(mmol/L)	11.58±5.7(mmol/L)	12.18±1. (mmol/L)
Ethanol	11.2±5.3(mmol/L)	10.64±3.7(mmol/L)	10.93±1.6(mmol/L)	9.63±5.6(mmol/L)
Water	10.3±6.3(mmol/L)	11.14±2.8(mmol/L)	8.79±4.9(mmol/L)	10.54±2.2(mmol/L)

Normal references(Giknis & Clifford, 2008) (7.1mmol to 11.7mmol)

Hematological Indices Outcome (Comparing Day 0 and Day 21)

The treated animals were fed with the CE extracts of water and ethanol, while the control groups were fed with only water and pellets from Unga Limited -Kenya. For the dosages, see *Table 2*.

The averages of red blood cells (RBC), White blood cells (WBC), Hemoglobin (Hb), Lymphocytes (LYMPH) Monocytes (MONO), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) of the five rats per group for day 0 in each group and day 21 for each group were done and compared.

There was a slight increase in RBC counts, with reference to rats exposed to ethanolic extracted extracts and so to the control groups, but a slight decrease in those exposed to water extracted extracts. All the changes were within normal ranges.

Hemoglobin values in both the groups increased (rats exposed to water extracted extracts and rats exposed to ethanolic extracted extracts) but a slight decrease was seen in control groups.

With reference to WBC counts, treated animals witnessed a decrease while controls saw an increase. The changes witnessed in lymphocytes and monocytes, in both treated animals and controls were within the normal ranges. With regards to hematocrit percentages, an increase was seen in treated animals a slight decrease in controls. See Table 5 below.

Table 5: Effects of Carissa *edulis* Water and Ethanolic Extracts on Hematological Parameters

Blood parameter	***Normal references (Giknis & Clifford, 2008)***	***DAY 0***			***DAY21***		
		Control	***Water***	***Ethanol***	***Control***	***Water***	***Ethanol***
RBC (10e6/µL)	***7.62-9.99***	*7.60±0.4*	*7.90±0.86*	*6.91±3.86*	*8.51±1.2*	*6.72±2.7*	*7.74±1.01*
Hb (g/dL)	***13.6-17.4***	*15.15±0.8*	*13.64±1.2*	*15.23±0.9*	*14.94±2.4*	*16.56±1.5*	*15.43±0.6*
WBC (10e3/µL)	***1.98-9.99***	*7.90±2.9*	*7.91±3.27*	*9.75±8.65*	*8.95±5.2*	*5.43±3.8*	*9.52±1.2*
LYMPH (%)	***44.7-87.1***	*45.10±18.6*	*73.62±3.28*	*65.22±16.77*	*44.94±2.3*	*56.02±6.9*	*75.3±0.2*
MONO (%)	***1-3.6***	*2.44±18.9*	*3.35±0.8*	*1.49±0.63*	*2.25±4.9*	*3.57±1.4*	*2.33±2.5*
HCT (%)	***38.5-49.2***	*54.14±3.49*	*44.95±2.6*	*46.32±2.77*	*51.07±5.3*	*47.15±6.4*	*50.34±4.2*
MCV (fL)	***46.3-56.2***	*62.70±3.51*	*59.58±4.13*	*57.82±2.87*	*57.67±2.8*	*56.67±1.7*	*61.77±3.2*
MCH (pg)	***16.3-19.5***	*21.56±1.6*	*19.32±0.63*	*19.32±1.26*	*18.27±1.4*	*21.74±0.8*	*21.55±1.04*
MCHC (g/dL)	***31.9-38.5***	*34.68±1.49*	*32.54±2.38*	*33.36±1.02*	*31.54±1.9*	*38.42±0.45*	*34.83±2.89*

Biochemical Outcome (Comparing Day 0 and Day 21)

Regarding biochemical parameters, an average was also made for the five rats for day 0 and day 21 and compared. Urea increased in the rats in all the treated animals and controls at the 21day, while creatinine increased in controls but decreased in ethanolic, and no change was seen with water extracts. Controls recorded a slight drop in ALTs (*Alanine Transaminase)* but slightly increased in both water and ethanolic extracted extracts. Regarding ASTs (*Aspartate aminotransferase*), there was a slight increase in both controls and treated animals. *See Table 6*.

Table 6: Biochemical changes of male wister albino rats exposed to water and ethanol extracts of Carissa *edulis*

Biochemical parameter	Normal references (Giknis & Clifford, 2008)	DAY 0			DAY 21		
		Control	Ethanol	Water	Control	Ethanol	Water
Urea mg/dl	10.7-20	4.87±5.2	7.91±6.9	5.05±1.2	11.8±4.5	22.5±4.3	17.5±6.3
Creatinine U/L	46-1230)	40.45±1.2	58.01±1.8	49.12±8.6	59.7±3.2	42.02±2.3	49.01±1.6
ALT's µ/L	19-48	43.47±6.2	29.50±2.9	34.02±5.3	48.9±4.3	42.5±4.8	41.5±2.2
ASTs µ/L	63-175	69.17±1.3	73.50±4.3	88.05±2.3	73.7±7.3	77.05±2.2	104.03±4.7

DISCUSSION

This study investigated the hematological and biochemical changes/responses of male Wistar albino-white *Rattus norvegicus* rats that were exposed to *Carissa edulis* ethanolic and aqueous extracts for 21 days (sub-chronic exposure). The herb was chosen based on previous studies that showed that *Carissa edulis* is not only used for the management of diabetes in most rural communities in Kenya and other part of the world but also in the management of other ailments; like abdominal ailments, epilepsy, and anemia (Chebor et al., 2020; Ibrahim et al., 2015).

But before exposing the herb to the rats, the herb had to be extracted, and its phytochemicals, determined. Inherent substances that would make a herb toxic, among many other factors could be due to its' chemical compounds -phytochemicals (Rodriguez-Fragoso et al., 2008), despite the fact, of its anti-diabetic activities. Saponnins for example, have been found to activate adenosine monophosphate protein- kinase enzyme **(**AMPK), in calcium-independent channels, regulating gluconeogenesis and glucose uptake (El Barky et al., 2017), and alkaloids has also been found to increase glucose absorption by body tissues (Kooti et al., 2016), though they might also be toxic. Hence the need for screening.

With regards to the dosages given to the rats- The amounts used was arrived, based on previous studies of CE ethanolic extracts. One such study was that of Ngulde et al., which found that a dose of 3,807.9 mg/kg would kill 50% (Ngulde et al., 2013) of the rats exposed to it. On calculation, we realized that, this would mean an approximate dosage 800mg of CE would kill 50% of the rats weighing an average weight of 200mg, but we needed a dosage that would produce, the least deaths or observable minimal effects, on the rats, hence the dosages given (*17.3 mg/100 g -ethanol and* aqueous extracts, 59*.1 mg/100 g).*

On exposure of the determined dosages to the rats, with regards to hematology, the hemoglobin levels in treated animals showed an increase, though slightly, and a slight decrease in controls. However, the change was insignificant. A finding signifying that ingestion of this herbal extract at the dose

that was given does not have any significant effect on the Hb, a finding similar to the study by (Jorum et al., 2016a; Ngulde et al., 2013). A mixed outcome was seen in RBC counts-while rats exposed to water extracted extracts slightly increased, those exposed to ethanolic extracted extracts decreased. Nevertheless, all the increases and decreases were within the normal ranges, a finding in agreement with the studies of Ngulde (Ngulde et al., 2013). With regards to hematocrit percentages, an increase was seen in treated animals and a slight decrease in was seen in the controls. All changes were also within the normal ranges.

In reference to WBC counts, treated animals witnessed a decrease while controls saw an increase. The changes witnessed in lymphocytes and monocytes, in both treated animals and controls were within the normal ranges, a finding that may indicate antimicrobial activity of these herb, other than being antidiabetic. This could explain why it has been used in management of various bacterial related ailments(Jorum et al., 2016b; Njau et al., 2017).

With regards to biochemical parameters, in all treated animals (ethanol and water extracted extracts groups), urea values showed an increase at the 21 days. Creatinine decreased in rats exposed to ethanolic extracted extracts, and no change was seen with those exposed to water extracted extracts. There was an insignificant drop in ALTs in control groups, but a slight increase was witnessed in both water and ethanolic extracted extracts groups. Concerning ASTs, there was a slight increase in control groups, but an insignificant increase was seen in both water and ethanolic extracted extracts groups. All these changes witnessed were within the normal ranges (Giknis & Clifford, 2008) , a finding that may indicate that, the oral administration of ethanolic and water extracts of CE, root, and bark did not alter the hepatocytes and kidneys function of the rats, a finding incongruence with that of (Ya'u et al., 2013).

CONCLUSSION AND RECCOMENDATIONS

Carissa edulis aqueous and ethanolic extracts, given by oral gavage once a day, to male Wistar albino rats, does not induce significant interference to the hematological and biochemical parameters, though, much more studies in a large scale (use of more rats).

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