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Full Length Research Paper

Antimalarial pyronaridine resistance may be associated with elevated *MDR-1* gene expression profiles but not point mutation in *Plasmodium berghei* ANKA isolates

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The selection of resistance is inevitable whenever chemotherapy is necessary for pathogen control. Notably, *Plasmodium falciparum* has developed multifaceted means to overcome the toxicity of nearly all antimalarial medicines. To bypass this challenge, not only should novel drugs be developed, but the resistance mechanisms to new and existing drugs need should be fully explored. Pyronaridine is a companion drug in Pyramax®, a blend of artesunate (ASN)-pyronaridine (PRD) which is the WHO prequalified alternative for malaria treatment in the African setting. However, half-life mismatch predisposes the PRD to swift emergence of resistance especially in high malaria transmission settings. However, there are no well-characterized PRD-resistant parasite lines. Previously, stable PRD- resistant *P. berghei* ANKA lines were selected by *in vivo* drug pressure and preliminary results showed cross-resistance with quinolines, therefore, hypothetically the activity of PRD and chloroquine or other quinolines may be comparable, hence, the resistance mechanisms may be parallel. Consequently, genetic polymorphisms and expression profiles of *PbMDR-1* that could be associated with pyronaridine resistance were examined by PCR amplification, sequencing and transcript quantification by RT-qPCR. The transcripts level increased during resistance selection while translated PbMDR-1 sequence alignment of PRD-sensitive and PRD-resistant was the same, the expression may be linked to PRD resistance but not mutations.

Key words: Quinolines, malaria, Pyronaridine, Pyramax®, resistance, expression, MDR-1 gene.

INTRODUCTION

Malaria is one of the most devastating infectious diseases faced by the humanity in the 21st century. Over the past two decades, numerous strains of *Plasmodium falciparum* have developed resistance to nearly all anti-

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malarial drugs presented for clinical treatment of malaria illness (Hanboonkunupakarn and White, 2015; Menard and Dondorp, 2017).This development has prompted a quest for new effective anti-malarial compounds with the least side effects (Tang et al., 2020). One such strategy for plummeting the malaria prevalence is the usage of twin- or triple-anti-malarial drug combinations, which is thought to protect each drug from the development of resistance and reduce the overall transmission of malaria (Dipanjan et al., 2017; Tse et al., 2019; Mishra et al., 2017). In the last 20 years, over 60 countries and territories have officially adopted artemisinin-based combination therapy (ACT) for the treatment of *falciparum* malaria (WHO, 2017). The artemisinin derivatives cause a rapid and effective reduction in parasite biomass as well as gametocyte carriage, while the partner drug, which has a longer duration of action, achieves effective clinical and parasitological cure. Despite evaluation of different forms of ACT (Henrich et al., 2014; Mishra et al., 2017), clinical failures or at least longer parasite clearance times have been described in western Thailand, southern Myanmar, and possibly in the Vietnam and Cambodia (Kyaw et al., 2013; Hien et al., 2012). This emergence of parasite resistance to some forms of ACT indicates that novel compounds and combinations must be discovered and developed (Ouji et al., 2018).

To overcome drug resistance challenge, several forms of Artemisinin Combinational Therapies (ACTs) have been evaluated and now the currently WHO prequalified combination therapy, PRD/ASN (Ashley and Phyo, 2018; Tse et al., 2019; Henrich et al., 2014), which deployment is under a pharmacovigilance system in countries that may consider the drug in their national treatment guidelines (WHO, 2020). Pyronaridine (PRD) is the bis-mannich base, an analog of amodiaquine and like lumefantrine, it has been found to act through the inhibition of β-haematin formation although the mechanisms remain unclear (Chang et al., 1992; Croft et al., 2012). Recently, attention has been renewed in pyronaridine as a likely partner for use in artemisinin-based combination therapy (ACT) for malaria treatment (Croft et al., 2012; Tse et al., 2019). Indeed, PRD is highly effective against CQ-sensitive and CQ resistant parasites (Gupta et al., 2002; Vivas et al., 2008). Although PRD has not been commercially available as monotherapy or extensively deployed outside China, the *in vitro* sensitivity to this drug decreased in China between 1988 and 1995, suggested the emergence of PRD resistance (Croft et al., 2012). Pyronaridine resistance has previously been selected in *Plasmodium berghei* and *Plasmodium yoelii* (Croft et al., 2012; Kimani et al., 2014; Peters and Robinson, 1992); however, the molecular organization of the phenotype was not investigated. Pyramax® (PRD/ASN), remains active against widespread Chloroquine (CQ) and Sufadoxine-Pyrimethamine (SP) drug-resistant *P. falciparum* clearance of malaria infection, studies indicate that use of drug combination with mismatched pharmacokinetics does not prevent selection of the resistance against long-acting drug (Hastings and Hodel, 2014; Li and Hickman, 2015). Artesunate is a short-acting artemisinin derivative with half-life of less than 2 h, while PRD is long-acting with half- life of 16 to 17days (Park and Pradeep, 2010). Indeed, PRD which is left trailing in sub-therapeutic doses within the body provides strong selection pressure for fast resistance emergence. Thus the need to understand the mechanism of PRD resistance towards the elucidation of molecular surveillance and health policy tools before or during drug use.

Single and multiple mutations, elevated transcript profiles and increased copy numbers of the *P. falciparum* multidrug resistance gene 1 (Pf *PbMDR-1*) have been linked with most antimalarial drug resistance in *P. falciparum* (Kiboi et al., 2014; Pradines et al., 2010; Tang et al., 2020). Therefore, to evaluate the role of point mutation and expression profiles in antimalarial pyronaridine resistance, we used previously generated stable PRD- resistant *P. berghei* ANKA lines by *in vivo* drug pressure (Kimani et al., 2014). Documented studies hypothesize that PRD mode of action may be comparable to that of CQ or other quinolines drug; consequently, they may share similar modes of action and resistance mechanisms (Dorn et al., 1998; Hanboonkunupakarn and White, 2015). As a result, the goal of this study is to identify genetic polymorphisms and expression profiles that could be associated with PRD resistance in selected genes associated with quinoline or any other anti-malarial drugs- preliminarily the multi-drug resistant gene 1 (*MDR-1*). This study is aware that the selected gene may not certainly be accompanying PRD resistance, thus proposes employment of whole-genome shotgun (WGS) sequencing approach and whole transcriptome profiling (WTP) of the dilution cloned resistant parasite using next sequencing generation sequencer (Illumina) to identify novel genes and copy number variation that may have accumulated during the drug selection process.

MATERIALS AND METHODS

Parasites and experimental animals

The drug sensitive parasite lines of *P. berghei* ANKA acronymed as PRD^S was used as reference line, while PRD^R is as previously submitted to drug selection pressure and cloned as described in detail (Kimani et al., 2014). Male Swiss albino mice weighing 20-22 g out-bred at KEMRI, Animal house Nairobi, utilized for this study. The animals warehoused in the animal house in hard plastic cages, standard polypropylene, and placed on commercial rodent feeds and water *ad libitum*. Antimalarial PRD drug was newly made by dissolving it in a solvent consisting of 70% Tween-80 (d = 1.08 g/ml) and 30% ethanol (d = 0.81 g/ml) and successively diluted ten-fold with double distilled water. All experiment in this study involving use of animals was conducted in accordance with KEMRI guidelines, as well as, internationally accepted principles for

Table 1. List of generations from pyronaridine resistant lines with the 50% effective doses (ED_{50}) determined for each of the generation obtained at each drug selection passage determined by 4-Day suppressive test.

Passage No.	5th	10th	15th	20th
ED(mg/kg)	5.2	17.32	89.11	102.12
	4.8	19.08	76.97	95.87
	4.74	18.61	70.08	78.90
				97.13

laboratory animal use and care.

Dilution cloning of PRD resistant parasite

To generate genetically homogenous PRD^R parasites, four different generations from pyronaridine resistant lines (Table 1) were dilution cloned as detailed (Janse et al., 2004). Briefly, a mouse with parasitemia between 0.3 and 1% was selected as a donor mouse. Then, 5 µl of infected blood (parasitized red blood cells, RBCs) was collected from the tail of the mouse using µl of heparinized syringe and diluted in 1 ml of 1 × PBS buffer. The number of infected erythrocytes per 1 µl was estimated from 20 µl of diluted blood. The cell suspension was then diluted further with 1 × PBS buffer to an estimated final concentration of 0.5 parasites/200µl PBS. Fifteen mice were intravenously injected each with 200µl /mouse of parasitized RBCs. Cloning was considered effective when about fifth to half of the inoculated mice tested positive and bared a parasitemia of between 0.3 and 1% at day 8 post infection.

Determination of indices of PRD resistance

The stability of PRD resistant line was evaluated by: (i) measuring drug responses after making five drug-free passages and (ii) freeze-thawing of parasites from -80°C stored for a period of four weeks followed by the measurement of effective doses in the 4-Day suppressive test. Stable resistance was defined as the maintenance of the resistance phenotype when drug-selection pressure was removed for at least five passages in mice (Kimani et al., 2014). Acquisition of resistance was assessed after every five drug passages using standard 4-DT to confirm the response levels of the parasite to the pyronaridine compound. 4-DT permits the measurement of the ED_{50} and ED_{90}, as well as the index of resistance at the 90% levels (I_{90}). The indices of resistance (I_{90}) were defined as the ratio of the ED_{90} of the resistant line to that of the parent strain as described by Kimani et al. (2014). Resistance was classified into three categories based on an earlier work (Merkli and Richle, 1980): I_{90} = 1.0, sensitive, (2) I_{90} = 1.01-10.0, slight resistance, (3) I_{90} = 10.01-100.0, moderate resistance and (4) I_{90}> 100.0, high resistance.

Pyronaridine sensitivity profiles tests

To assess the resistance profile of individual clones generated by dilution cloning, the fastest growing clone among the four generation was selected and evaluated for its response to PRD in the 4-DT (Fidock et al., 2004). Briefly for each selected clone, mice were infected intraperitoneally with 1 × 10^6 parasites/mouse. Oral treatment of drug was initiated on day 0 (4 h post infection) and continued for 24, 48 and 72 h post infection. Parasite density was estimated microscopically (×100) after 96 h post parasite inoculation using thin blood films made from tail blood. Parasite growth was then followed for at least 15 days post-infection to assess the recrudescence of the parasites after cessation of drug treatment as earlier described elsewhere (Kiboi et al., 2014).

Parasite preparation and extraction of parasitic DNA

Parasitized blood cells were harvested from mice under general anaesthesia, when trophozoite stages were most prevalent into PBS (PH 7.2). In preparing parasitic DNA, mouse white blood cells was removed by successive filtration of infected blood using Plasmodipur filters (Euro-Diagnostica) as previously described (Janse et al., 2004). Intact parasites were released from their host red blood cells (RBCs) using RBC lysis buffer (Roche). Briefly, packed cells was re-suspended in 5 volumes of cold (4°C) erythrocyte lysis buffer for 5 min until the suspension becomes a clear red colour before spinning at 2000rmp for 8 min to pellet the parasite. Total genomic DNA of both the sensitive and resistant parasites was extracted using commercially available QiAamp DNA Blood kit (Qiagen) according to manufacturer's instructions.

Amplification and sequencing of *PbMDR-1* gene

A 4260-nucleotide length fragment of the*MDR-1* gene was amplified by Polymerase Chain Reaction (PCR). Briefly, *MDR-1* gene was amplified by PCR and sequenced using the primer pairs described by Kiboi et al. (2014) and also provided in Supplementary Table S1. The reaction mixture consisted of approximately 200 ng of genomic DNA, 0.5 µM of forward and reverse primers, 1X PCR buffer (Promega), 2 mM $MgCl_2$, 200 µM deoxynucleotide triphosphate (dNTP) and 0.025 U DreamTaq polymerase (Eurogentec) in a final volume of 25µ µl. The thermo-cycler (Applied Biosystems)) was programmed as follows: an initial 95°C for 5 min followed by 30 cycles of 95°C for 1 minute, 48°C for 30 s and 68°C for 5 min. A final 10-min extension step was done at 72°C. A *MDR-1* gene sequence was obtained from http://plasmodb.org. Accession No. PBANKA_123780. Primers for amplification and sequencing were manually designed (Table 1). Products of PCR amplification were analysed using gel electrophoresis system. After PCR products were purified by GeneJET™ PCR purification kit, sequencing was conducted using ABI Prism Big Dye Terminator v3.1 (Applied Biosystems, CA, and U.S.A) cycle sequencing ready reaction kits according to the manufacturer's instructions.

Evaluation of gene expression of *PbMDR-1*gene

To determine the expression profile of the *PbMDR-1* at specific selection stage presented as 5th, 10th, 15th and 20th drug pressure passage, clones were generated by dilution cloning and the best growing clone among the generations were selected as described earlier (Janse et al., 2004). To assess the mRNA transcripts quantity of *PMDR-1*gene, briefly fresh parasites were centrifuged and the pellet prepared. The total RNA was extracted from at least

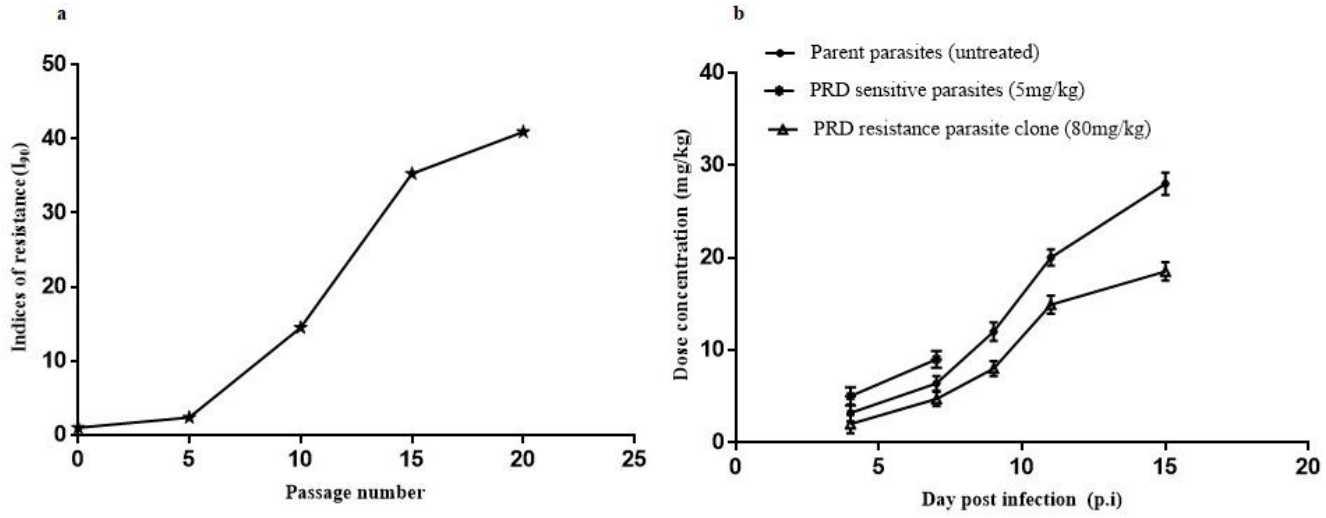

Figure 1a. The indices of resistance recorded during selection of antimalarial pyronaridine resistance determined by 4-Day suppressive test. (b) Growth profiles of the best growing clones generated by dilution cloning of the pyronaridine resistant parasite of 20th passage number (PRD^{R}) compared to the wild-type drug sensitive line (PRD^{S}). The data points were gotten from an averaging parasitaemias observed from tail blood film of five mice per group.

1×10^6 parasites based on High Pure RNA extraction kit (Roche™). The RNA was immediately used for cDNA synthesis following the Transcriptor First Strand cDNA synthesis kit (Roche™).Briefly, the Transcriptor Reverse Transcriptase (20 U/µl) were added to first strand cDNA and RT reaction mix was incubated at 50°C for 60 min, then at 85°C for 5 min and finally chilled on ice. The cDNA was used as template for RT-PCR assays or stored at −15 to −20°C for longer period. Primers (Supplementary Table S2) were designed to run the reactions for *PbMDR-1* in the same plate by using similar cycling conditions. Relative quantification of transcripts was performed using the housekeeping gene Pbβ-actin (PBANKA_145930) as a reference gene. The difference in relative expression levels of *PbMDR-1* was calculated from $2^{-\Delta\Delta Ct}$ value after normalization of data to actin (Livak and Schmittgen, 2001). All analyses were performed triplicate. The conditions for these reactions were: 94°C for 4 min; followed by denaturation at 94°C for 15 s and annealing/extension at 56°C for 30 s, for 40 cycles.

Data analysis

Gene sequences were manually compiled, then compared between drug selected and unselected clones (Altschul et al., 1990) and then analysed using MEGAX software (Kumar et al., 2018). The relative expression level data was normalized using Pbβ-actin as endogenous control gene using the formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

RESULTS AND DISCUSSION

Previously, stable PRD- resistant *P. berghei* ANKA lines were selected by *in vivo* drug pressure (Figure 1a). The fastest growing clone of PRD resistant line (PRD^{R}) was selected and subjected to drug sensitivity profiles (Table 1, Figure 1b). Subsequently, the best growing clone with high resistance profile was considered for the evaluation of genetic polymorphism and quantification of transcripts. Then the *MDR-1* coding sequence of both PRD-Sensitive and PRDR clones were amplified and sequenced (Supplementary Figure S1). To explore the potential modulatory and compensatory role of *MDR-1*, the mRNA transcript level was measured. Consequently, these results demonstrated that the transcripts level increased during selection of pyronaridine resistance (Figure 2), while the translated PbMDR-1sequence alignment of PRD-sensitive and PRD-resistant was 100% identical (Supplementary Figure S2). Consistence with part of *in vivo* results, Pradine et al. reported the absence of association between pyronaridine *in vitro* responses and polymorphisms in genes involved in quinoline resistance in *Plasmodium falciparum* (Pradines et al., 2010).

The global dissemination of drug-resistant *P. falciparum* is spurring intense efforts to implement artemisinin (ART)–based combination therapies for malaria, including ASN/PRD. Nevertheless, resistance to at least one component of some forms of ACT currently in clinical use has been documented, and it is feared that ACT will gradually lose its clinical efficacy due to widespread use. Individual *P. falciparum* parasites with longer clearance times have been described in Cambodia (Rogers et al., 2009).

The biggest challenge at present is genetic elasticity and high frequency of human malaria *parasite P. falciparum* to develop resistance to structurally and mechanistically related and unrelated drugs (accelerated resistance to multiple drugs, ARMD) (Rathod et al., 1997), ARMD is characterized by the ability of a strain to generate a drug-resistant clone when put under drug pressure. This results from the high mutation rate during

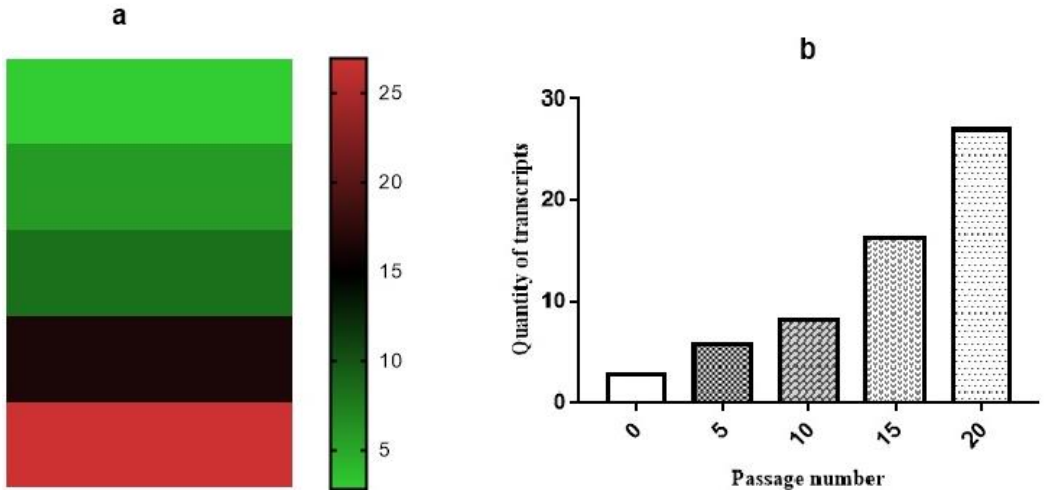

Figure 2. Expression profiles of multidrug resistance gene 1 (PbMDR-1) as measured from cDNA derived from total RNA isolated from dilution cloned Pyronaridine resistant lines at 5th ,10th 15th and 20th drug selection stages (Passage number) relative to their wild type drug sensitive parental clones (0th).

parasite multiplication. The goal of the present study was to investigate the role of multidrug resistance gene -1 which is known to be associated with reduced quinoline susceptibility in modulating PRD resistance. Previously, PRD resistance line was developed for over a period of six months and cloned the parasite which was highly growing and with the highest resistance; however, the mechanisms underlying this resistance was never investigated (Kimani et al., 2014).

It has been proposed that the selective pressure for resistance to PRD-ASN combinations would be exerted by the longer-acting PRD component. The mechanism by which resistance to pyronaridine develops is unknown, but may be due to a direct effect on the pyronaridine mechanism of action or modulation of targeted gene. For instance, Wu et al. (1988) described an increase in the number of food vacuoles in trophozoites from a pyronaridine-resistant *P. berghei* (RP) line, some of which were fusing. There was also a marked reduction in the digestive food vesicles containing malaria pigment granules for both trophozoites and schizonts and typical hemozoin grains were not formed in the pyronaridine-resistant parasites (Auparakkitanon et al., 2006; Wu et al., 1988). These and other ultra-structural differences suggested that resistance may be due to a direct effect on the pyronaridine mechanism of action. Another study by Li et al., found over-expression of a 54 kDa protein in a pyronaridine-resistant strain of *P. berghei* (ANKA) (Li et al., 1995). The protein was localized mainly in the cytoplasm of erythrocytic stage trophozoites, schizonts and merozoites and less commonly in the cytoplasm of infected erythrocytes(Li et al., 1995). Interestingly, a 54 kDa protein is also expressed in chloroquine-resistant *P. berghei* (ANKA) suggesting a common effect, though whether this is related to resistance development remains unknown (Li et al., 1995). Notably, Qi et al. proposed that antimalarial pyronaridine could be an inhibitor of P-glycoprotein mediated multidrug resistance in tumor cells (Qi et al., 2002; Qi et al., 2004).

The *P. falciparum* multidrug (MDR) resistance protein (PfMDR-1) has been implicated in altering parasite susceptibility to a variety of currently available antimalarial drugs. Point mutations in *PfMDR-1* have been associated with changes in parasite susceptibility to Chloroquine, Quinine, Mefloquine, and Artemesinin derivatives in both laboratory lines and clinical isolates; however, these mutations have limited use as molecular markers (Duraisingh and Cowman, 2005; Woodrow and Krishna, 2006).

Conclusion

Comparison of coding region of parent strain and pyronaridine resistant line revealed that the nucleotide and translated protein sequence of the PbMDR-1 was identical. Thus, point mutation in *MDR-1* was not involved in the generation of pyronaridine resistance. However, the expression levels of the gene increased in tandem with the increase of the resistance implying that the expression profile may be linked to PRD resistance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Table S1. The primer sequences used for PCR amplification and sequencing *PbMDR-1* candidate gene.

Primer name	Primer sequence (5′ to 3′): PCR primers
PbMDR-1-1F UTR	GTCTAAATGTTGTAATTTGTTGTCCT
PbMDR-1-R (UTR)	GACATTATCTAATTTCATCACCTTG
Primer name	Pbmdr-1: sequencing primer (5′ to 3′)
PbMDR-1-F (UTR)	TTCACGCTATAAAAGTACAGACTA
PbMDR-1-1R	CAGTATCATTCACACTTTCTCC
PbMDR-1-2F	GTGCAACTATATCAGGAGCTTCG
PbMDR-1-2R	CACTTTCTCCACAATAACTTGCTACA
PbMDR-1-3F	GCAGCTCTATATGTAATAAAAGGGTC
PbMDR-1-3R	GTCGACAGCTGGTTTTCTG
PbMDR-1-4F	CTTTGAATTACGGTAGTGGCT
PbMDR-1-4R	TCGCTAGTTGTATTCCTCTTAGA
PbMDR-1-5F	TGGAGTAGTTAGTCAAGATCCT
PbMDR-1-5R	GTGCCTTGTTCAACTATTACAC
PbMDR-1-6F	TCAAATAGAGATCAAGAATCAACAGG
PbMDR-1-6R	GGATATAAACCACCTGCCACT
PbMDR-1-7F	GCCAAGTAAACCATCATTCTTCA
PbMDR-1-7R	TCGCGTTGTAATGGTATATGCT
PbMDR-1-8F	GGATTTTTATCGTCGCATATTAACAG
PbMDR-1-8R	TAGCTTTATCTGCATCTCCTTTGAAG
PbMDR-1-9F	TGCAATAGATTATGACAGTAAAGGGG=-
PbMDR-1-9R	ATCTTTCAAATCGTAGAATCGCAT
PbMDR-1-10F	CTTCAAAGGAGATGCAGATAAAGCTA
PbMDR-1-10R	GATTCAATAAATTCGTCAATAGCAGC
PbMDR-1-11F	TGCAATAGTTAACCAAGAACCAATGT
PbMDR-1-11RUTR	CAATAGCCGATTAAAAGAAAAAACGA

Table S2. Real-time PCR Primers used to assess *PbMDR-1* transcription levels with Pbβ-actin as endogenous control.

Primer Name	Primer Sequence (5’ to 3’)	Position	Length	Tm	GC%
PbMDR-1-F	GACCCAACAGACGGAGATATTG	1282	22	62.2	50
PbMDR-1-R	GTCCACCTGACAACTTAGATGAG	1723	23	62.2	47.8
PbMDR-1-F	CTGTAGCAAGTTATTGTGGAGAAAG	716	25	61.9	40
PbMDR-1-R	CTCCGTCTGTTGGGTCATAAA	1297	21	61.8	47.6
PbMDR-1-F	TGGAGAAACTGGATGTGGTAAA	3471	22	61.8	40.9
PbMDR-1-R	AGGCTCTAGCAATAGCAACTC	3976	21	61.7	47.6
PbMDR-1-F	CAGGAGCTTCGTTGCCTATT	188	20	62.2	50
PbMDR-1-R	AGCCACTACCGTAATTCAAAGT	928	22	62.1	40.9
PbMDR-1-F	TTCTGATACAGGTGCTGCTAAA	2142	22	61.8	40.9
PbMDR-1-R	GGATATAAACCACCTGCCACTAT	2399	23	61.9	43.4
Pb β-actin -F	CAGCAATGTATGTAGCAATTCAAGC	392	24	61.8	56.8
Pb β-actin –R	CATGGGGTAATGCATATCCTTCATAA	523	25	61.7	58.9

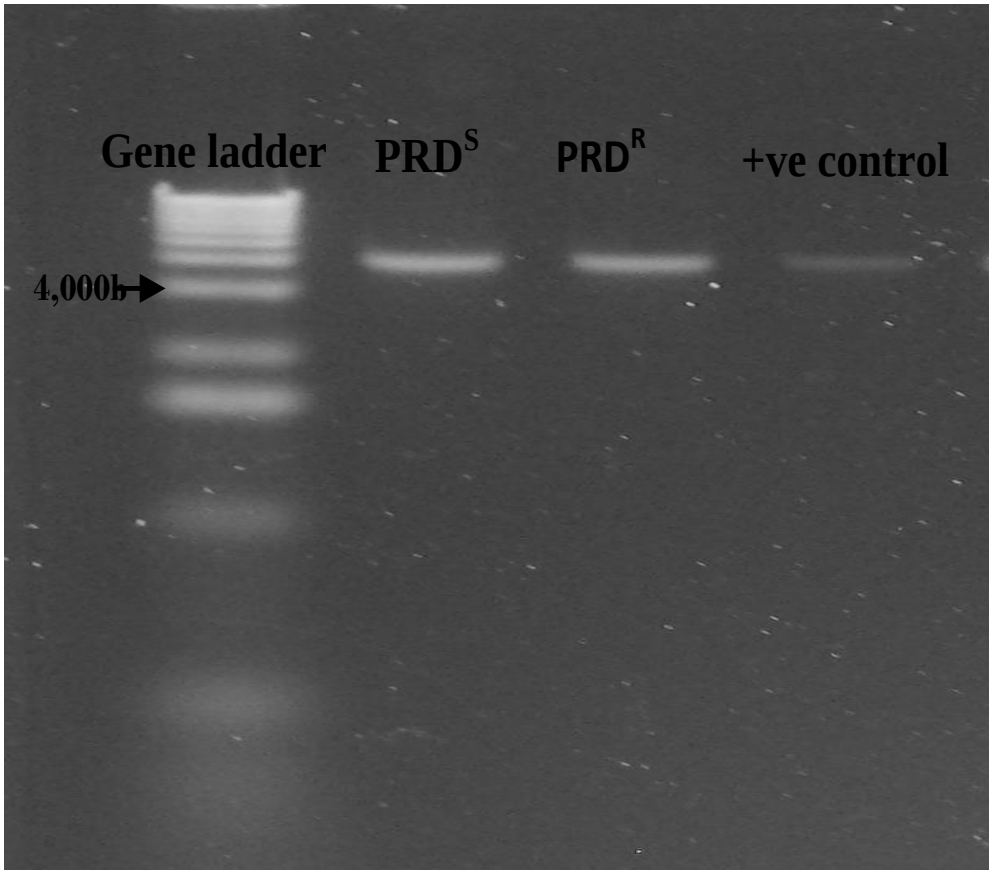

Figure S1. Gel photo of amplified mdr-1 gene isolated from both sensitive (PRD^S) and resistant clone (PRD^R) that was and run against gene ladder and positive control.

Figure S2. Pbmdr-1-S (Sensitive clone) and Pbmdr-1-R (Resistant clone) alignment by Clustal Omega.

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Sub-acute and protective effect of *Cymbopogon citratus* against carbon tetrachloride-induced liver damage

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The study evaluates the sub-acute toxicity and antioxidant potential of ethanolic leaf extract of *Cymbopogon citratus* against CCl_4-induced toxicity in Sprague Dawley rats. The ethanolic leaf extract of *C. citratus* was prepared by solvent maceration method. The phytochemicals present in the extract were determined using standard methods. The potential sub-acute toxicities were evaluated using OECD procedure. The sub-acute toxicity of the extract at the doses of 125, 250 and 500 mg/kg, b.wt. was administered orally for 28 days. Another sets of rats were made hepatotoxic by orally administered with CCl_4 (20% CCl_4 in olive oil) twice per week for a period of five weeks. They were treated with *C. citratus* extract (300 and 600 mg/kg body weight) once a day for 35 days. Biochemical parameters were used to assess the hepatoprotective effects of the extract on liver tissues. Phytochemical screening of *C citratus* shows the presence of anthraquinones, alkaloids, flavonoids, etc. The administration of *C. citratus* is not hematotoxic and significantly reduced ($P<0.05$) elevated liver biomarker enzymes, urea, creatinine and the level of malondialdehyde. Treatment with the extract was found to significantly increase ($P<0.05$) TP level, the activities of superoxide dismutase and catalase. Liver histopathology shows that the extract reduced the incidence of liver lesions induced by CCl_4. The administration of *C. citratus* did not produce any toxic effects in the sub-acute study. The plant exhibits potent protective effects in CCl_4-induced liver damage due to decrease in liver biomarker enzymes activities, increase of antioxidant-defense system and inhibition of lipid peroxidation.

Key words: Sub-acute toxicity, protective effects, *Cymbopogon citratus,* carbon tetrachloride, hematological, oxidative stress parameters.

INTRODUCTION

Cymbopogon citratus is prominent and commonly used in alternative medicine for the treatment of diverse ailments. *C. citratus* is a tropical monocotyledonous hypogeal perennial herb belonging to the family Poaceae and is commonly known as lemon grass. Several bioactive compounds have been reported to be isolated from the plant. The oil from *C. citratus* plant is used as culinary flavoring, scent, and medicine. Citronelle compound obtained from *C. citratus*, acts as an antihypertensive agent by inducing vasodilatation of vascular smooth muscles (Bastos et al., 2010; Chitra et al., 2012). Furthermore, citral obtained from the plant has been shown to possess activities like antiproliferative effect against *Trypanosoma cruzi* (Santoro et al., 2007),

antiparasitic effects against leishmaniasis (Santin et al., 2009; Oliveira et al., 2009), anti-mutagenicity (Vinitketkumnuen et al., 1994) and antinociceptive (Viana et al., 2000). *C. citratus* effectively treats fever, infection, headaches, rheumatic pain, nervous and digestive disorders. The plant also acts as a sedative, antispasmodic, analgesic, and anti-inflammatory agent (Naik et al., 2010; Figueirinha et al., 2010). In Nigeria, lemon grass is used to treat fever, jaundice, hypertension, diabetes mellitus and obesity (Adeneye and Agbaje, 2007).

Hepato-toxicity is a method used in animal model, for liver damage investigation for screening the hepato-protective activity of natural medicinal plant. The use of natural products for liver diseases is growing because of their safety and efficacy as an alternative remedy compared with chemically synthesized drugs (Natanzi et al., 2009). Histo-pathological changes in liver tissue; activities of alkaline phosphatase (ALP), gamma glutamyltransferase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH); levels of malondialdehyde (MDA), reduced glutathione (GSH) and other related parameters are used to assess liver toxicity and the hepato-protective activity of medicinal plants (Kumar et al., 2009; Uboh et al., 2012).

Liver helps in detoxification of drugs, exogenous toxins and therapeutic agents; it also helps in the bio-regulation of amino acids, proteins, carbohydrates, fats, blood coagulation and immunomodulation (Juza and Pauli, 2014). Impairment of the liver generally occurs from excessive exposure to toxicants, chemotherapeutic agents, alcohol, protozoan and viruses (Juza and Pauli, 2014). Experimental model used to induce liver damage in animals is by using carbon tetrachloride (CCl_4). CCl_4 is activated by cytochrome (CYP) 2E1, CYP2B1 or CYP2B2 and possibly CYP3A, to form the trichloromethyl radical (CCl_3^-) (Slater, 1984). This radical can bind to cellular molecules (protein, lipid, nucleic acid), impairing crucial cellular processes such as lipid metabolism, which results in fatty acid degeneration (steatosis) (Raucy et al., 1993). CCl_3^- forms adducts with DNA, which initiate the onset of hepatocellular carcinoma. This radical can also react with oxygen to form the trichloromethylperoxy radical CCl_3OO^-, which is a highly reactive species. The substance (CCl_3OO^-) reacts with polyunsaturated fatty acids and phospholipids to initiates the chain reaction of lipid peroxidation reaction.

This affects the permeabilities of mitochondrial, plasma membranes and endoplasmic reticulum resulting in the loss of cellular calcium sequestration and homeostasis, which may contribute heavily to subsequent cell damage (Weber et al., 2003; Mehendale et al., 1994). CCl_4 intoxication is mediated by two types of nonparenchymal liver cells, viz., Kupffer and stellate cells. The activation of Kupffer cells by CCl_4 mediate inflammatory processes via the nuclear factor kappa B (NF-kB) signal transduction pathway with production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6(IL-6) and other inflammatory mediators; cyclooxygenase-2 (Cox-2) and inducible nitric oxide synthase (iNOS) (Gallucci et al., 2000; Gruebele et al., 1996), which in turn causes full activation of the mitogen activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) and the Janus kinase (Jak)-signal transducer and activator of transcription protein (STAT) pathway. These pathways are involved in the regulation of cell proliferation and apoptosis (Bak et al., 2016). Stellate cells are normally quiescent and fat-storing cells, but after activation by agents like CCl_4, they display a typical acute-phase response (Nieto et al., 2000), take on a fibroblast like appearance, release nitric oxide, begin to overproduce type-I collagen and thus promote hepatic fibrosis (Lee et al., 1995).

The efficient potency of *C. citratus* on free radical scavenging and other reactive oxygen species and antioxidation ability led us to evaluate the sub-acute toxicity and the protective effect of ethanolic leaf extracts of *C. citratus* on carbon tetrachloride-induced liver damage in male Sprague Dawley rats.

METHODOLOGY

Collection and identification of plant material

The leaves of *C. citratus* were obtained from Ikorodu in Lagos State, Nigeria. The plant was authenticated by a botanist from the department of Botany, University of Lagos, Lagos, Nigeria. Authentication number for *C. citratus* was given (6946).

Preparation of ethanolic leaf extract of *C. citratus*

The leaves of *C. citratus* were washed, air dried under shade in the Biochemistry Laboratory, pulverised to coarse power using blender. Extraction was carried out by dispersing 200 g of the ground *C. citratus* plant material in 1 L of 90% ethanol and shaking was done with GFL shaker for 72 h. This was followed by vacuum filtration and concentrated by rotary evaporator at a temperature not exceeding 40°C. The concentrated extract was dried to complete dryness in an aerated oven at 40°C for 48 h. The extract was later stored in a refrigerator at 4°C.

Phytochemical analysis of ethanolic leaf extract of *C. citratus*

Phytochemical tests for bioactive constituents were carried out on

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small portions of the plant extract using standard phytochemical procedures (Trease and Evans, 1986; Sofowora, 1993; Kokate, 1994).

Experimental animals

A total of 70 male Sprague Dawley albino rats with body weight ranging from 200 to 220 g were obtained from Ratzmattazz Nigeria enterprises, 21 insurance estate satellite town, Lagos, Nigeria. They were acclimatized for two week to laboratory condition of 23±2°C. They were kept in plastic cages and fed with commercial rat chow and supply with water *ad libitum.* The rats were used in accordance with NIH Guide for the care and use of laboratory animals; NIH Publication revised (2011).

Sub-acute toxicity test

The sub-acute toxicity test was conducted in accordance with the guidelines published by the Organization for Economic Cooperation and Development (OECD, 2007) No. 407 with slight modification. At the onset of dosing, the rats weighed 210 ± 10 g each. Twenty eight acclimatized rats were grouped into four groups. Each group contains seven animals. Group I served as the positive control group and received distilled water, for 28 consecutive days, while the other groups (II, III and IV) received a daily amount of 125, 250 and 500 mg/kg b.wt. of ethanolic leaf extract *C. citratus* orally, for 28 consecutive days, respectively. Food and water intake were given freely. After 28 days of the feeding trial, the rats were fasted overnight (for at least 20-24 h) before they were sacrificed.

Body weight determination

The individual body weights of all animals were recorded weekly (7 days interval) during the course of the sub-acute toxicity study. The body weights were also recorded prior to testing and terminally (after fasting) prior to when they were sacrificed.

Administration of CCl_4

Male albino rats (Sprague Dawley) of about sixteen weeks old with weight range of 200 to 220 g were made hepatotoxic by orally administered with CCl_4 (20% CCl_4 in olive oil) dosage of 1 ml/kg body weight twice per week for a period of five weeks according to the method described by Momoh et al. (2018a). The animals were all treated once per day according to the grouping of the animals as shown in the following. Forty two acclimatized rats were grouped into six groups. Each group contains seven animals as follows: Group A-Normal control; Group B-Negative control (CCl_4 without treatment); Group C-Positive control (CCl_4 + 100 mg/kg b.wt. silymarin); Group D-Olive oil only; Group E-CCl_4 + 300 mg/kg b.wt. of *C. citratus* leaf extract; Group F-CCl_4 + 600 mg/kg b.wt. of *C. citratus* leaf extract.

Collection of blood samples

All the albino rats were sacrificed by cervical decapitation after 20-24 h fasting. Blood was collected from the albino rats by ocular puncture into EDTA tubes for hematological analysis and the remaining blood was collected in heparinised tubes and centrifuge at 3000 rpm for 20 min and the plasma stored at -20°C to estimate biochemical parameters. The animals were dissected while their livers and kidneys were excised for biochemical and histological examinations.

Determination of hematological parameters

The hematological parameters were determined in the whole blood using BC-3200 Auto Hematology Analyzer in University of Lagos Teaching Hospitals (LUTH) in Idi-Araba, Lagos, Nigeria. The hematological parameters investigated were as follows: White blood cell count (WBC), Monocyte number (Mid#), Monocyte percent (Mid%), Granulocyte number (Gran#), Granulocyte percent (Gran%), Lymphocyte number (Lym#), Lymphocyte percent (Lym%), Hemoglobin (HGB), Red blood count (RBC), Hematocrit (HCT), Mean cell volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), Red Blood Cell Distribution Width Coefficient of Variation (RDW-CV), Red Blood Cell Distribution Width Standard Deviation (RDW-SD), Platelet count (PLT), Mean platelet volume (MPV), Platelet Distribution Width (PDW) and Plateletcrit (PCT).

Measurement of plasma liver biomarker enzymes and lipid profile

Liver damage was assessed by the estimation of plasma activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), total protein (TP), total cholesterol (TC), triglyceride (TG), high density lipoprotein-cholesterol (HDL-Chol), were measured using commercially available test kits from Randox Laboratories Ltd. (UK). LDL- Cholestrol was calculated according to Momoh et al. (2018b). LDL-C=TC - HDL-C - TG/5. Kidney damage was assessed using urea and creatinin Randox kits.

Hepatic antioxidant activities

Preparation of liver homogenate

The liver tissues of some of the sacrificed albino rats were excised and the liver samples were cut into small pieces and homogenized in phosphate buffer saline (PBS) to give a 10% (w/v) liver homogenate. The homogenates were then centrifuged at 12,000 rpm for 50 min. The supernatant obtained was later used for assay of thiobarbituric acid reactive substances (TBARS) content, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and reduced glutathione (GSH).

Determination of lipid peroxidative (LPO) indices

Lipid peroxidation as evidenced by the formation of TBARS was measured in the homogenate by the method of Jiang et al. (1992).

Determination of superoxide dismutase (SOD)

The SOD activity was estimated by its capacity of inhibiting pyrogallol autooxidation in alkaline medium. The liver homogenate was assayed for the presence of SOD by utilizing the technique described by Zou et al. (1986).

Determination of catalase (CAT)

The liver homogenate was assayed for catalase colorimetrically at 620 mm and expressed as µmoles of H_2O_2 consumed/min/mg protein by the method of Rukkumani et al. (2004).

Determination of reduced glutathione (GSH)

Reduced glutathione (GSH) was determined in the liver

homogenate using the method of Rukkumani et al. (2004).

Determination of glutathione peroxidase (GPx)

Reduced glutathione (GSH) was determined in the liver homogenate using the method of Rukkumani et al. (2004).

Histopathological studies

The histopathological analyses were assayed in the Department of Anatomy, College of Medicine, University of Lagos, Idi-Araba, Lagos, Nigeria. The albino rats were sacrificed and their abdomens were cut open to remove their liver and kidney. Some of the organs were fixed in Boucin's solution (mixture of 75 ml of saturated picric acid, 25 ml of 40% formaldehyde and 5 ml of glacial acetic acid) for 12 h, and then embedded in paraffin using conventional methods (Galighor and Kozloff, 1976). They were cut into 5 µm thick sections and stained using haematoxylin-eosin dye and finally mounted in di-phenyl xylene. The sections were then observed under microscope for histopathological changes in the liver and kidney architecture and their photomicrographs were taken.

Data analysis

The results were calculated and expressed as Mean ± Standard deviation. Data analyses were done using the GraphPad prism computer software version 5.01. One-way analysis of variance (ANOVA) was used for comparison for determining the significant difference. The inter group significant was analysed using Posthoc Turkey's and Bonferroni's multiple comparison test. A *P*-value < 0.05 was considered significant.

RESULTS

Phytochemical screening of ethanolic leaf extract of *C. citratus*

Phytochemical screening of ethanolic leaf extract of *C. citratus* shows the presence of secondary metabolite like tannins, steroid, anthraquinones, triterpenoids and saponin (Table 1).

Sub-acute toxicity study

Clinical observations and survival of animals administered with C. citratus

The study shows no mortalities were recorded in the rats over the period of 28 days of treatment with *C. citratus* leaf extract at the doses of 125, 250 and 500 mg/kg, b.wt., through oral gavage. None of the animals after administration of *C. citratus* at the doses of 125, 250 and 500 mg/kg, b.wt., showed any obvious morbidity or clinical symptoms of toxicity such as changes in the eyes, skin and fur, autonomic (salivation, perspiration and piloerection), stereotype activities and respiratory rate problem throughout the experimental period of 28 days.

Body weight determination of experimental animals

The body weight of the animals administered with the plant extract were recorded at an interval of 7 days over the treatment period of 28 days and there were significant increase ($P<0.05$) in the body weight of the animals administered with the plant extract at different concentrations when compared with the healthy control group (Figure 1). The increase in the body weight for all groups was mostly dose dependent as a greater increase in body weight was observed in high dose group.

The effect of *C. citratus* ethanolic leaf extract on liver biomarker enzymes and lipid profile in male albino rats

There were significant reduction ($P<0.05$) in AST activity, LDL-Chol and creatinine levels in animals administered *C. citratus* extract (groups III and IV) compared to group I animals. ALT and GGT activities, TC and TG levels did not show any significant different ($P>0.05$) in all the rats administered *C. citratus* extract for the sub-acute toxicity test when compared with the non-treated animals (group I). The plasma total protein (TP) concentration and HDL-Chol was significant increased ($P<0.05$) in the treated group (groups II-IV) animals compared to group I animals (Table 2).

Sub-acute toxicity test

The oral administration of *C. citratus* leaf extract (125, 250 and 500 mg/kg b.wt.) in sub-acute toxicity study showed no toxic sign or death of rats after 28 days. Animals administered *C. citratus* extract showed significant increase ($P<0.05$) in catalase (group III), GPx (group IV) SOD% (group III and IV), SOD units (groups II - IV) and TP (groups III and IV) in their liver homogenate while MDA values reduces significantly ($P<0.05$) in groups II to IV animals when compared with group I rats (Table 3).

Sub-acute histological study

The histological study for the kidney and liver are as shown in Figure 2.

Hematological analysis

Table 4 shows that there were significant increase ($P<0.05$) in WBC, Mid#, Mid%, Gran%, Gran#. MCH, HGB, HCT, MCHC, RBC and their Lymph# and Lymph% were significantly lowered ($P<0.05$) in the animals treated with *C. citratus* leaf extract compared to the animals administered with CCl_4 without treatment. The animals in group A showed significant increase ($P<0.05$) in Lymph#,

Table 1. Phytochemical screening of ethanolic leaf extract of *Cymbopogon citratus*.

Phytochemical constituent	Test performed	Inference
Tannins	Ferric chloride test	+
Saponins	Froth test	+
Antraquinone	Borntrager's test	+
Alkaloids	Dragendorff's test	+
	Mayer's test	+
	Wagner's test	+
Flavonoids	Ferric chloride test	+
	Shinoda test	+
Steroids and sterol	Liberman Burchard test	+
	Salkowski's test	+
Triterpenoids	Sulphuric acid test	+
Phenolic compounds	Ferric chloride test	+
Anthocyanine	Sodium hydroxide test	+
Carbohydrate	Benedict's test	+
	Fehling's test	+
	Molisch's test	+

(+) Present.

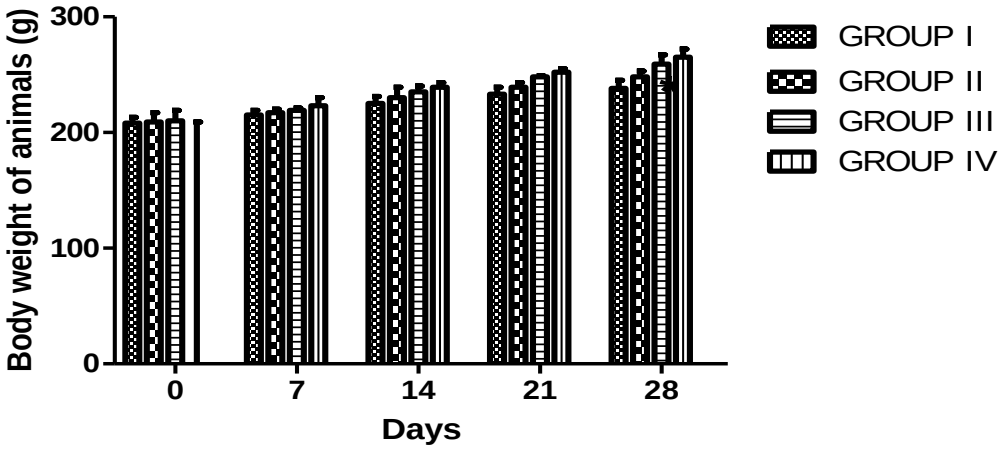

Figure 1. The effect of different concentration of *Cymbopogon citratus* leaf extract on body weight of male Sprague Dawley rats.

MCV and decrease (P<0.05) in Mid%, Gran%, HGB, MCH, and PLT when compared with animals administered with *C. citratus* extract (Table 4).

Analysis of liver biomarker enzymes and lipid profile

There were significant (P<0.05) increase in liver biomarker enzymes (AST, ALT, ALP and GGT), urea and creatinine in group B untreated animals compared to all other animals in other groups. Group B animals also have lower level of TP value compared to healthy animals (group A) and animals administered with *C. citratus* extract (Table 5).

Determination of oxidative stress parameters

Oxidative stress parameters (SOD% inhibition, SOD unit, CAT and GSH) were significantly (P<0.05) reduced in

animals administered with CCl_4 without treatment compared to the control group animals (group A) and animals treated with *C. citratus* extract. The MDA values of group B rats were significantly ($P<0.05$) increased compared to other groups (Table 6).

Histopathological studies

The liver architecture of the healthy animal, animal infected with CCl_4 without treatment and animals treated with *C. citratus* extract are as shown in Figure 3.

DISCUSSION

Phytochemical screening of ethanolic leaf extract of *C. citratus* shows the presence of secondary metabolite like flavonoids, alkaloids, tannins, steroid, anthraquinones, triterpenoids, saponin, etc. (Table 1). The presence of these secondary metabolites in *C. citratus* may be responsible for the antioxidant and protective properties of the plant. Studies have shown that reactive oxygen species (ROS) are not only responsible for oxidative stress at low levels, they are also considered to play an important role in normal cell physiological functions, acting as modulators of redox regulated processes (Droge, 2002; Schreck and Baeuerle, 1991). These ROS are continuously produced during normal physiologic events and normally removed by antioxidant defence mechanisms (Zorov et al., 2006; Chen et al., 2006). Plants are potential sources of antioxidants, since synthetic antioxidants have side effects when consumed *in vivo* (Ghasemzadeh and Ghasemzadeh, 2011). Polyphenols (total phenolic, flavonoids and proanthocyanidin contents) are the major plant compounds with antioxidant activity. This antioxidant activity is believed to be mainly due to their redox properties (Zheng and Wang, 2001), which play an important role in adsorbing and neutralizing free radicals, decomposing peroxides, quenching singlet and triplet oxygen. The results from this study strongly suggest that phenolics are important components of these plants, and some of their pharmacological effects could be attributed to the presence of these important secondary metabolites.

General behavioral changes in body weight are preliminary indicators of early signs of toxicity caused by various drugs and chemicals (Ezeja et al., 2014). The body weight of the animals administered with *C. citratus* extract increases significantly ($P<0.05$) when compared with the control group and was considered normal. Thus, it can be concluded that *C. citratus* oral administration did not produce any major clinical toxicological signs and did not affect the normal growth pattern of the animals throughout the treatment period of 28 days.

In toxicity rating by joint FAO/WHO Expert Committee on Food Additives (WHO,1966), if at 2 g/kg oral dose of a substance causes no death, it is sufficient to assume that the substance is relatively non-toxic. The sub-acute toxicity study shows that the plant extract of *C. citratus* is non-toxic and no mortality was observed in all the groups. The calculated LD_{50} value was greater than 500 mg/kg b.wt. The kidney is susceptible to damage caused by various toxic substances as large volume of blood flows through it and the toxins filtered usually gets concentrated in the kidney tubules (Al-Attar et al., 2017). Clinical biochemistry analysis was conducted to investigate any possible influence of the extract on hepatic and renal functions of the rats. Biochemical parameters are considered as an important marker for toxicity evaluation, as both liver and kidney are necessary for the survival of an organism (Suganthy et al., 2018). The extract did not damage the liver as evidenced by significant decreased ($P<0.05$) in the level of plasma activity of AST (group III). ALT, GGT, TC, and TG did not show any significant difference while plasma concentration of TP and HDL-C (groups III and IV) significantly increases ($P<0.05$) in the animals administered with the extract (Table 2). Increase of these transaminases (AST, ALT and GGT) in the plasma is an indication of necrotic lesions within the liver. AST and ALT are mainly used to detect injury to liver cells (hepatocytes). Under normal circumstances, these enzymes (AST and ALT) reside in the hepatocytes. However, these enzymes will leak into the blood stream if the liver is injured, thus raising their levels in the blood (Oriakhi et al., 2018). In a research work carried out by Eraj et al. (2016) aqueous extract of C. citratus was administered at a dose of 200 mg/kg body weight orally for 15 days to healthy rabbit. The extract exhibited significant reduction in biochemical parameters (ALP, SGOT, SGPT, GT and TB) as observed in their study (Eraj et al., 2016). GGT acts as an indicator for cholestasis (e.g. biliary duct obstruction). Obstructed bile duct will induce the synthesis of GGT, thus elevating the levels in the blood (Bulle et al., 1990). The animals administered C. citratus extract (groups III and IV) had significant ($P<0.05$) reduced creatinine level compared to group I animals. Serum creatinine level is a good indicator of renal function since elevation of serum creatinine level is associated to a marked failure of nephron functions (Lameire et al., 2005). The study shows that the plant does not have toxic effect on the kidney.

Oxidative stress is caused by the presence of ROS in excess of the available of antioxidant buffering capacity. Many studies have showed that ROS can damage proteins, lipids and DNA, thus altering the structure and function of the biological cell, tissue, organ and system, respectively (Momoh et al., 2018a). Catalase catalyzes the conversion of hydrogen peroxides into oxygen and water and protects the tissue from oxidative damage by highly reactive oxygen free radicals and hydroxyl radicals

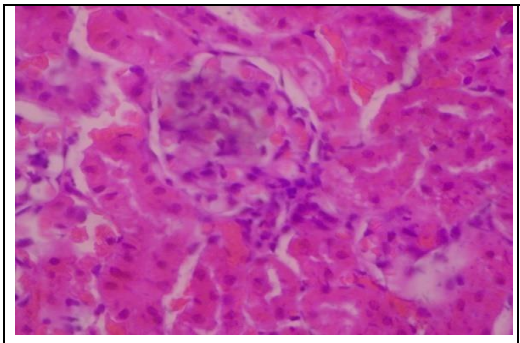

Plate 1 Selected photomicrograph of kidney section (x400) in group I (control) showing normocellular glomerular tufts disposed on a background containing renal tubules with no visible lesions.

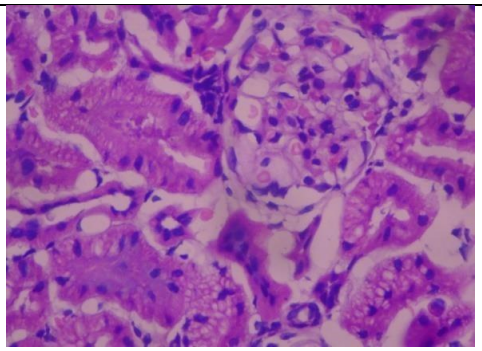

Plate 2 Selected photomicrograph of kidney section (x400) in group II (125 mg/Kg. bwt) showing normocellular glomerular tufts disposed on a background containing renal tubules with no visible lesions.

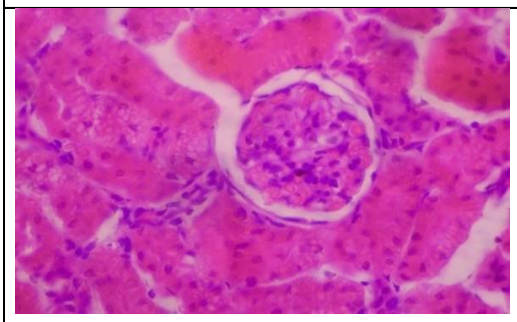

Plate 3 Selected photomicrograph of kidney section (x400) in group III (250 mg/Kg. bwt) showing normocellular glomerular tufts disposed on a background containing renal tubules with no visible lesions.

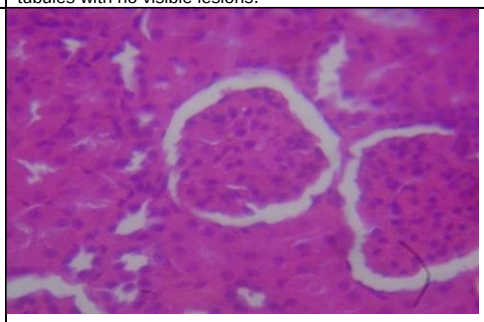

Plate 4. Selected photomicrograph of kidney section (x400) in group IV (500 mg/Kg. bwt) showing normocellular glomerular tufts disposed on a background containing renal tubules with no visible lesions.

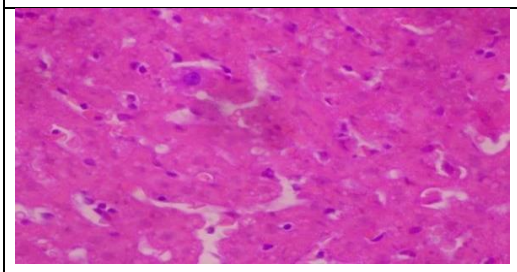

Plate 5. Selected photomicrograph of liver section (x400) in group I (control) showing normal hepatocytes with no visible lesions.

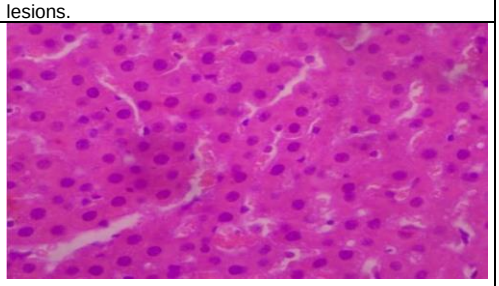

Plate 6. Selected photomicrograph of liver section (x400) in group II ((125 mg/Kg. bwt) showing normal hepatocytes with no visible lesions.

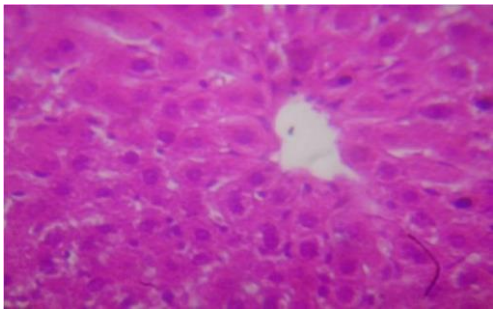

Plate 7. Selected photomicrograph of liver section (x400) in group III (250 mg/Kg. bwt) showing normal hepatocytes with no visible lesions.

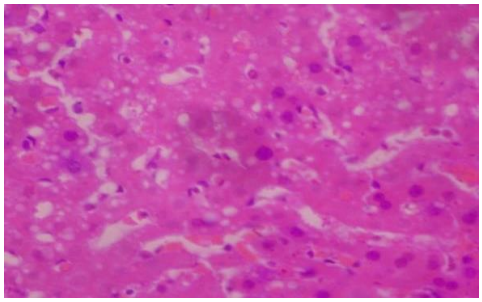

Plate 8. Selected photomicrograph of liver section (x400) in group IV (500 mg/Kg. bwt) showing normal hepatocytes with mild fatty deposit.

Figure 2. Photomicrograph of kidney and liver section stained with hematoxylin and eosin (H&E ×400) for sub-acute toxicity test with animals administered with *Cymbopogon citratus*.

Table 2. The effect of *Cymbopogon citratus* ethanolic extract on liver biomarker enzymes and lipid profile in albino rats.

Parameter	Group I	Group II	Group III	Group IV
AST (U/L)	18.53±1.09^{ab}	17.72±1.05^{b}	15.66±1.13^{c}	20.05±1.25^{a}
ALT (U/L)	9.57±1.04^{ab}	8.05±0.85^{b}	10.82±1.16^{a}	10.20±1.56^{a}
GGT (U/L)	2.22±0.74^{ab}	1.26±0.52^{b}	2.68±0.39^{a}	2.95±0.76^{a}
TC (mg/dl)	107.82±9.43^{a}	112.54±9.32^{a}	109.63±8.79^{a}	101.81±2.15^{a}
TG (mg/dl)	72.91±9.61^{a}	83.62±17.55^{a}	81.43±10.17^{a}	73.98±18.24^{a}
HDL-Chol (mg/dl)	58.08±2.42^{c}	61.76±2.32^{bc}	64.17±2.69^{b}	69.13±3.42^{a}
LDL-Chol (mg/dl)	35.16±1.29^{a}	34.06±2.08^{a}	29.17±1.15^{b}	17.88±1.07^{c}
TP (mg/dl)	9.55±1.02^{b}	11.56±0.80^{a}	11.94±0.36^{a}	12.07±0.41^{a}
Creatinine (mg/dl)	0.758±0.086^{a}	0.784±0.095^{a}	0.497±0.079^{b}	0.479±0.088^{b}

Data are presented as Mean ± SD (n=7). One-way ANOVA Posthoc Tukey's test was used for comparing significant difference between the different groups across the rows. a=highest, b= medium, c=lowest. Those groups that have the same letters are not significant (P>0.05) while those that have different letters are significant (P<0.05) when comparing across the rows.

Table 3. The effect of *Cymbopogon citratus* ethanolic extracts on oxidative stress parameter in Sprague Dawley albino rats.

Parameter	Group I	Group II	Group III	Group IV
MDA (nmol/l)	7.07±0.852^{a}	5.45 ±0.473^{b}	5.25 ±0.391^{b}	4.32±0.685^{c}
Catalase (µmol/min/mg protein)	71.75±1.65^{b}	72.34±1.72^{b}	74.87±1.36^{a}	74.01±1.42^{ab}
GSH	0.285±0.078^{a}	0.296±0.087^{a}	0.264±0.077^{a}	0.312±0.068^{a}
GPX	0.374 ±0.009^{b}	0.424±0.007^{ab}	0.394±0.071^{ab}	0.441±0.019^{a}
(SOD)% inhibition	89.79±1.56^{b}	92.53±1.87^{ab}	93.08±2.89^{a}	93.45 ±1.81^{a}
SOD unit	8.79±0.883^{c}	12.39±0.927^{b}	13.85±0.657^{a}	14.27±0.842^{a}
Total protein (g/dl)	6.13±0.25^{b}	6.92±0.48^{b}	8.67±0.78^{a}	8.43 ±0.60^{a}

Data are presented as Mean ± SD (n=7). One-way ANOVA Posthoc Tukey's test was used for comparing significant difference between the different groups across the rows. a=highest, b= medium, c=lowest. Those groups that have the same letters are not significant (P>0.05) while those that have different letters are significant.

(Momoh et al., 2018a). Glutathione (GSH) is a dipeptide compound containing glutamate, cysteine and glycine amino acids whose antioxidant function is facilitated by the sulphydryl group of cysteine. In the oxidation reaction of glutathione, the sulphur forms a thiyl radical that reacts with a second oxidized glutathione forming a disulphide bond (GSSG). GSH is found in most plant and animal tissues, cells and subcellular compartments of higher plants. GSH can reacts chemically with superoxide, singlet oxygen and hydroxyl radicals and therefore function directly as a free radical scavenger. Glutathione may stabilize membrane structure by removing acyl peroxides formed by lipid peroxidation reactions (Price et al., 1990). Glutathione peroxidase is a selenium-dependent enzyme, which decomposes H_2O_2 and various hydro- and lipid peroxides (Kinnula et al., 1995). SOD is an effective defence enzyme that catalyzes the dismutation of superoxide anions into hydrogen peroxide (Momoh et al., 2018a). We observed significant increase (P<0.05) in catalase, SOD%, SOD unit and total protein in the animals' administered *C. citratus* compared to the control healthy animals in the sub-acute toxicity test. The level of GSH and GPX (except for group IV) did not show any significant difference while MDA values were lower in the rats administered with *C. citratus*. This is an indication that the plant can reduce oxidative stress caused by the presence of ROS.

Histological study shows that the tissue shows normocellular glomerular tufts disposed on a background containing normal renal tubules and no abnormalities are seen in Plates 1 to 4. Plates 5 to 7 histopathology of the liver shows normal radially arranged hepatocytes extending from portal tracts to central veins and no fatty change or sinusoidal congestion are seen but Plate 8 shows small cytoplasmic fat microvesicles (Figure 2). Sub-acute administration of *C. citratus* did not cause any major toxic effects on the biochemical parameters, liver and kidney architectures. The hepato-protective effects of *C. citratus* extract in rats with oxidative stress induced by CCl_4 was investigated. It is generally believed that the hepatotoxicity induced by CCl4 is due to the formation of the active metabolite, trichloromethyl free radical ($CCl_3^{\bullet}$). This then readily interacts with molecular oxygen to form the trichloromethyl peroxy radical ($CCl_3OO^{\bullet}$). Both

Table 4. Hematological parameters of CCl_4-induced hepatotoxic rats treated with silymarin and *Cymbopogon citratus* extracts.

Hematological parameter	Group A	Group B	Group C	Group D	Group E	Group F
WBC ($\times10^9$/L)	13.66±3.02$^{nn'}$	11.29±1.06$^{n'o}$	13.46±0.74$^{nn'}$	16.06±0.99^{mn}	18.15±2.73^{m}	14.5±1.11^{n}
Lymph# $\times10^9$/L	5.12±0.421$^{mm'}$	4.70±0.616$^{mm'}$	4.36±0.545$^{m'n'}$	5.41±0.414^{m}	2.73±0.553^{n}	3.72±0.247$^{n'}$
Mid# $\times10^9$/L	1.32±0.035^{o}	0.71±0.059^{q}	3.51±0.181^{m}	2.14±0.154^{n}	1.13±0.083^{p}	1.38±0.066^{o}
Gran# $\times10^9$/L	3.82±0.125^{m}	1.70±0.045^{o}	1.82±0.055^{o}	3.18±0.073^{n}	3.77±0.093^{m}	3.71±0.094^{m}
Lymph%	47.34±2.05^{n}	66.90±3.28^{m}	49.56±3.24^{n}	51.18±3.08^{n}	38.68±2.28^{o}	49.43±2.52^{n}
Mid%	9.45±0.722^{p}	9.20±0.831^{p}	11.52±0.688^{o}	15.18±0.852^{n}	16.45±1.131^{n}	18.55±1.077^{m}
Gran%	44.52±2.06^{n}	23.93±0.95^{o}	43.35±2.59^{n}	44.14±1.79^{n}	50.75±1.63^{m}	48.18±0.87^{m}
HGB g/dl	15.98±0.65^{n}	11.91±0.42^{p}	15.04±0.67^{no}	14.00±0.85^{o}	18.18±0.70^{m}	17.63±0.90^{m}
RBC ($\times10^9$/L)	7.98±0.27$^{on'}$	6.33±0.13^{q}	7.15±0.23^{pq}	7.27±0.30^{op}	9.32±0.74^{m}	8.49±0.82$^{nn'}$
HCT%	47.68±2.91$^{nm'}$	44.01±1.46^{o}	48.98±1.98$^{nm'}$	47.88±1.97$^{nm'}$	52.55±1.10^{m}	50.60±1.50$^{mm'}$
MCVfl	66.92±1.69^{m}	62.81±3.31$^{mm'}$	67.06±3.74^{m}	64.91±4.29^{m}	58.01±3.57$^{m'n}$	56.30±5.32^{n}
MCH pg	20.24±0.64^{n}	18.47±0.93^{n}	19.03±0.76^{n}	18.96±0.71^{n}	32.90±2.33^{m}	33.43±2.54^{m}
MCHC g/dl	30.84±0.89$^{m'n}$	29.51±0.71^{n}	29.32±1.63^{n}	29.11±0.93^{n}	33.35±1.42^{m}	32.48±1.19$^{mm'}$
RDW-CV %	17.56±1.835^{m}	16.90±0.530^{m}	16.52±0.462^{m}	16.98±0.510^{m}	15.33±1.201^{m}	15.98±1.810^{m}
RDW-SD fl	38.67±3.98^{m}	36.31±2.70$^{mm'}$	37.28±2.39$^{mm'}$	37.06±2.15$^{mm'}$	33.54±2.94$^{mm'}$	32.72±3.87$^{m'n}$
PLT ($\times10^9$/L)	501.23±39.31$^{n'}$	685.31±25.28^{m}	561.29±23.25$^{m'nn'}$	672.20±51.73^{m}	601.48±45.98$^{m'n}$	624.87±41.84$^{mm'}$
MPV fl	8.26±0.913^{m}	7.91±0.341^{m}	8.11±0.133^{m}	7.66±0.775^{m}	8.13±0,422^{m}	7.68±0.765^{m}
PDW	16.46±1.181^{m}	16.53±0.945^{m}	16.16±1.067^{m}	16.28±0.847^{m}	16.65±0.684^{m}	16.13±0.667^{m}
PCT %	0.416±0.041$^{nn'}$	0.507±0.033^{m}	0.449±0.021$^{mn'}$	0.516±0.098^{m}	0.506±0.052$^{mn'}$	0.505±0.043$^{mn'}$

The values are the Means ± SD for seven rats in each group. Values with different alphabet superscript in the same row indicate significantly different (P<0.05). Comparisons across the row were done using Bonferroni's multiple comparison. A P<0.05 was considered statistically significant.

Table 5. Effect of silymarin and *Cymbopogon citratus* extracts on plasma liver biomarker enzymes, urea, creatinine and TP in CCl_4-induced hepatotoxic rats.

S/N	Parameter	Group A	Group B	Group C	Group D	Group E	Group F
1	AST (U/L)	18.55±1.22^{n}	80.27±8.71^{m}	24.73±3.65^{n}	23.01±4.52^{n}	23.24±2.53^{n}	22.51±3.14^{n}
2	ALT (U/L)	10.78±1.11^{o}	48.35±4.45^{m}	25.61±3.93^{n}	30.05±4.83^{n}	15.69±1.49^{o}	13.52±2.89^{o}
3	ALP (U/L)	8.96±0.94^{o}	24.32±3.12^{m}	9.43±1.62^{o}	13.38±2.74^{n}	10.17±2.76^{no}	8.23±2.17^{o}
4	GGT (U/L)	2.29±0.18$^{n'}$	10..25±0.46^{m}	3.75±0.17^{n}	3.16±0.27$^{nn'}$	2.67±0.45$^{n'}$	2.77±0.67$^{n'}$
5	Urea (mg/dl)	19.83±1.85^{p}	34.87±2.11^{m}	29.34±1.19^{n}	25.42±1.71^{o}	30.73±1.67^{n}	32.22±2.86^{mn}
6	Creatinine (mg/dl)	0.743±0.09^{n}	0.897±0.02^{m}	0.801±0.01^{n}	0.798±0.03^{n}	0.839±0.02^{n}	0.644±0.07^{o}
7	TP (g/dl)	9.65±0.87^{n}	7.66±0.53$^{n'}$	8.84±0.94$^{nn'}$	8.25±0.65$^{n'}$	11.75±0.42^{m}	11.49±0.82^{m}

The values are mean ± S.D, for seven rats in each group. Values with different alphabet superscript in the same row indicate significantly different (P<0.05). Comparisons across the row were done using Bonferroni's multiple comparison. A P<0.05 was considered statistically significant.

Table 6. Effect of silymarin and *Cymbopogon citratus* extracts on oxidative stress parameters in CCl_4-induced rats.

Oxidative stress parameter	Group A	Group B	Group C	Group D	Group E	Group F
LPO ($\times10^3$ mM MDA/mg protein)	8.26±0.19$^{n'o}$	16.56±2.30^{m}	10.11±0.92$^{nn'}$	11.45±1.15^{n}	6.73±0.39^{op}	5.87±1.25^{op}
CAT (µmol/min/mg protein)	71.83±6.85^{m}	45.63±3.93^{o}	68.36±6.83$^{mm'}$	59.70±5.46$^{m'n}$	73.54±5.93^{m}	70.81±4.73^{m}
SOD% inhibition	90.61±4.85^{m}	58.74±3.45^{o}	82.52±3.22$^{m'}$	70.14±4.26^{n}	85.36±3.81$^{mm'}$	87.62±3.07$^{mm'}$
SOD unit	9.65±2.95^{m}	1.42±0.28^{p}	4.72±0.96$^{m'o}$	2.35±0.32^{op}	5.83±0.95$^{m'n}$	7.08±1.87$^{mm'}$
GSH (mg/mg protein)	0.37±0.01^{m}	0.16±0.09$^{pp'}$	0.26±0.02^{no}	0.21±0.01$^{op'}$	0.29±0.02$^{m'n}$	0.35±0.05$^{mm'}$

The values are mean ± S.D (n = 7). Values with different alphabet superscript in the same row indicate significantly different (P<0.05). Comparisons across the row were done using Bonferroni's multiple comparison. A P<0.05 was considered statistically significant.

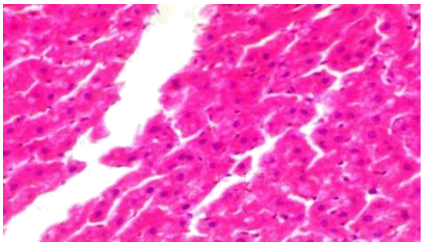

Plate 9. Selected photomicrograph of liver sections stained with hematoxylin and eosin (H&E ×400) for group A (control) showing normal histological structure of hepatocytes, hepatic cords, central vein and sinusoids.

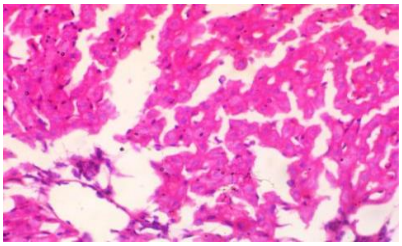

Plate 10. Selected photomicrograph of liver sections stained with hematoxylin and eosin (H&E ×400) for rat administered with CCl_4 and olive oil (group B) mixture. Inflammation of cells and degeneration of hepatocytes due to necrosis were observed.

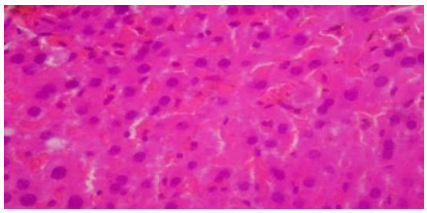

Plate 11. Selected photomicrograph of liver sections stained with hematoxylin and eosin (H&E ×400) for group C animals showing packing of the hepatic sinusoids with red blood cells and congestion.

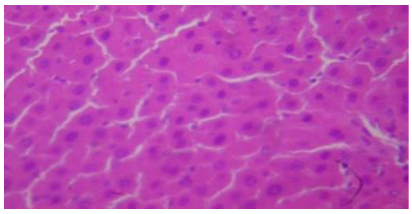

Plate 12. Selected photomicrograph of liver sections stained with hematoxylin and eosin (H&E ×400) for group D rats administered with olive oil.

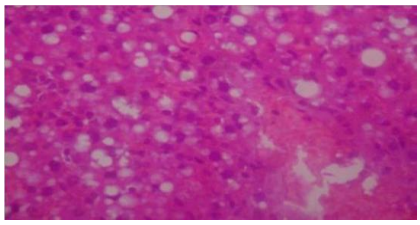

Plate 13. Selected photomicrograph of liver sections stained with hematoxylin and eosin (H&E X 400) for group E rats treated with 300 mg/kg b.wt of *C. citratus* leaf extract for five weeks showing many hepatocytes contain cytoplasmic fat vacuoles.

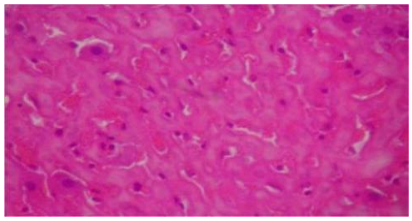

Plate 14. Selected photomicrograph of liver sections stained with hematoxylin and eosin (H&E ×400) for group F rats intoxicated with CCl_4 and treated with 600 mg/kg b.wt of *C. citratus* extract. No abnormalities seen.

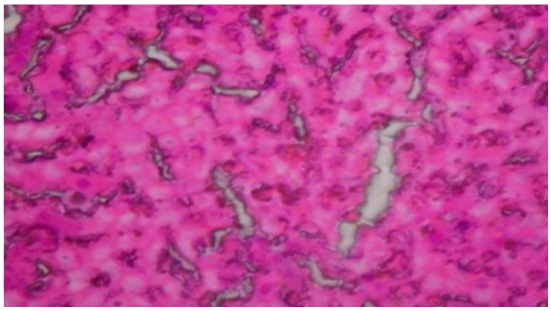

Plate 15. Selected photomicrograph of liver sections stained with hematoxylin and eosin (H&E X 400) for group F rats intoxicated with CCl_4 and treated with 600mg/Kg b.wt of *C.citratus* extract. No abnormalities seen.

Figure 3. Photomicrograph of liver section stained with hematoxylin and eosin (H&E X 400) for CCl_4-induced liver damage in male albino rats treated with silymarin and *Cymbopogon citratus* extracts.

radicals are capable of binding to lipids, proteins and other macromolecules with simultaneous attack on poly-unsaturated fatty acids to produce lipid peroxidation leading to hepatotoxicity (Momoh et al., 2018a). H_2O_2 have also been used as an animal model for the induction of liver damage (Mello et al., 1984; Ganie et al., 2011).

Hematological and biochemical indices are reliable parameter for the assessment of the health status of animals (Momoh et al., 2018a). Evaluation of hematological parameters would be helpful in determining the toxic effects of *C. citratus* extract on animal blood. WBC helps the body to fight against infection, defend the body by phagocytosis against invasion by foreign organisms and to produce or at least to transport and distribute antibodies in immune response. RBC helps to check the level of anemia and to evaluate normal erythropoiesis. HGB level shows the amount of intracellular iron present while HCT indicates the volume of RBC in 100 ml of blood and it helps to determine the degree of anemia or polycythemia (Momoh et al., 2018a). The study shows that there are significant decrease ($p<0.005$) in the level of blood WBC, Mid#, Mid%, Gran%, Gran#, HGB, RBC, HCT, MCH and MCHC of the CCl_4 intoxicated rats (Group B) compared to the animal treated with *C. citratus* extract (Table 4). The significant reduction ($P < 0.05$) in these hematological parameters in Group B animals may be attributed to the cytotoxic effects and suppression of the erythropoiesis caused by the administration of CCl_4. There were significant increase ($P<0.05$) in the Lymph#, Lymp% in the animals administered CCl_4 without treatment compared with animals treated with *C. citratus* extract (groups E and F). *C. citratus* extract causes significant increase ($P<0.05$) in Mid%, Gran%, HGB, MCH, PLT, and a decrease in Lymph# and MCV values in groups E and F rats compared to healthy animals (group A). This is an indication that the plant may aid in the increase of the immune system against infections and stimulate the production of hemoglobin. Other hematological parameters like RDW-CV, RDW-SD, MPV, and PDW showed no significant differences in the entire groups. The results obtained from this study showed clearly that ethanolic leaf extracts of *C. citratus* is not hematotoxic.

The present study demonstrates that *C. citratus* extract attenuates liver damage due to CCl_4 administration as indicated by the significant reduction in the elevated levels of AST, ALT, ALP, GGT and increase in TP levels of groups E and F animals. The administration of *C. citratus* extract displayed similar results as that of the control (group A), with slight amelioration in most of the studied parameters. The result obtained from this study showed that there were significant increase ($P<0.05$) in the levels of AST, ALT, ALP and GGT values of group B animals compared to other animals in other groups. This may imply that severe damage occurs in the liver cells of the animals administered with CCl_4 since the activities of these enzymes are reported to be increased in liver damage. Treatment with *C. citratus* extract and silymarin markedly reduced the effect of CCl_4 induced liver damage as evidenced by decreased in the level of these plasma liver biomarker enzymes activities (AST, ALT, ALP and GGT). The significant increase in these liver biomarker enzymes in the plasma of these animals is an indication of hepatotoxicity of the liver in the animals administered with CCl_4 (Mahesh et al., 2009) and this causes cellular leakage and loss of functional integrity of the hepatic cell membrane (Gupta and Singh, 2007; Kalegari et al., 2014). The study shows that there were significant increase ($P<0.05$) in the urea and creatinine levels of group B animals compared to other groups. This is an indication of severe kidney damage in group B animals. Group B rats have lower level of TP value compared to healthy animals (group A) and animals administered with *C. citratus* extract. The significant decrease ($P<0.05$) in the total protein values of animals administered with CCl_4 without treatment compared to other animals in groups A, C, E and F, respectively, may be due to considerable liver damage through induction of peroxidation of lipids and inhibiting protein synthesis due to trichloromethyl free radical covalent bindings (Momoh et al., 2018a; Lee et al., 2004). In this study, there was significant increase ($P<0.005$) in the catalase, SOD% inhibition, SOD unit and decrease in MDA values in the liver tissue homogenate of the rats treated with *C. citratus* extract and group A animals compared with group B animals. MDA increased after oral administration with CCl_4, treatment with *C. citratus* leaf extracts and silymarin reduce the level of MDA ($P<0.05$). Inhibition of elevated MDA levels observed in *C. citratus* extract and silymarin treated groups may be due to their antioxidant and free radical scavenging activities through re-establishment of biomembranes of hepatic parenchymal cells. Nwosu's study shows that aqueous leaf extract of *C. citratus* exhibits protective role in animals exposed to toxic dose of paracetamol by its ability to enhance free radical scavenging activity which lead to increase in the levels of antioxidants measured (Nwosu et al., 2015). Furthermore, it was observed that aqueous leaf extracts of *C. citratus* has an antihepatotoxic action against dimethylnitrosamine (DMN) induced hepatic oxidative damage in rats which might be ascribed to its antioxidant and free radical scavenging property (Naglaa et al., 2015). The observed protective effect of silymarin against lipid peroxidation could be related to its antioxidant effects which assist in the preservation of membrane integrity. Silymarin can chelate transition metal ions such as copper and iron rendering them effective antioxidants (Momoh et al., 2018a).

The results of the histological study are as shown in Figure 3. Histological examination results are consistent with that of the biochemical analysis. The liver of the control animals (group A) showed a normal arrangement of hepatocytes and sinusoids. The cytoplasm was not

vacuolated. Areas of infiltration by inflammatory cells, changes in fats and necrosis were not observed (Plate 9). Group B rats, which were exposed to CCl_4 for 35 days, exhibited severe histo-pathological alterations which include cytoplasmic vacuolization, inflammation of cells, congestion, infiltration, and degeneration of hepatocytes due to necrosis (Plate 10). The rats (group C) treated with silymarin showed sinusoidal congestion (Plate 11). The group D rats showed normal arrangement of hepatocytes and sinusoids, the olive oil did not affect the liver architecture of the animals. Histologic section of tissue shows parallel plates of hepatocytes with oval nuclei and moderate eosinophilic cytoplasm. All the vessels appear normal; no abnormalities are seen (Plate 12). The group E rat showed many hepatocytes containing cytoplasmic fat vacuoles (Plate 13), while animals administered with higher concentration of *C. citratus* (group E) showed significant improvement evident through a well arranged of hepatocytes with cytoplasm not vacuolated (Plates 14 and 15). Sinusoids well preserved, no fat inclusions or atypia is seen and no abnormalities seen when compared with CCl_4 intoxicated rats without treatment (Figure 3). In another research work carried out by Naglaa et al. (2015), it was observed that animals administered with *C. citratus* significantly reversed the effect of dimethylnitrosamine on the liver structure in the histopathological study (Naglaa et al., 2015). This study shows that *C. citratus* ethanolic leaf extracts significantly ($P<0.05$) reduces the damage effect of CCl_4 on liver architecture of male Sprague Dawley rats.

Conclusion

The current results demonstrate that *C. citratus* has a potent hepatoprotective effect against CCl_4-induced liver injury in Sprague Dawley rats. *C. citratus* treatment significantly reduced increase in liver biomarker enzyme activities and attenuates oxidative stress-induced pathological changes

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Insulin resistance and specific biomarkers in blood and urine of type 2 diabetic patients with or without nephropathy in Basrah, Iraq

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Diabetic nephropathy (DN) is a master cause of all surplus death-rate among type 2 diabetes mellitus (T2DM) patients with microalbuminuria. This study aimed to find effective biomarkers for early predicting of DN. Present study included 63 patients with T2DM (31 patients with DN, 32 patients without DN) and 33 healthy controls. These three groups were matched for their glucose, urea, creatinine, insulin, L-Carnitine (LC), osteoprotegerin (OPG), sialic acid (SA), trace elements (Selenium, Zinc, Magnesium), albumin (Alb), and fibronectin (FN). Glucose, urea, and creatinine were determined by spectrophotometer. Insulin, LC, OPG, SA, Alb, and FN were assayed by enzyme-linked immunosorbent assay (ELISA). Insulin resistance (IR) was calculated by the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) equation. Selenium was measured by hydride generation while Zinc and Magnesium were measured by flame atomic absorption spectrometer. Compared with controls, the results indicated that T2DM patients with or without DN had a significant increase in glucose, urea, creatinine, insulin, IR, OPG, SA, Alb, FN and a significant decrease in LC and trace elements levels. It was concluded that IR is strongly associated with obesity and had an important role in the pathogenesis and increased complication of diabetes which could be used as excellent indicators for early-stage DN in T2DM patients and thus decreasing mortality and morbidity.

Key words: Diabetic nephropathy, insulin resistance, obesity, oxidative stress, traces elements.

INTRODUCTION

Diabetic nephropathy (DN) is defined as the manifestation of incessant clinical albuminuria in a person with diabetes (for more than 5 years) and accompanying retinopathy in absence of urinary tract infection, other kidney diseases and heart failure. DN is a multi-stage state that takes many years to be clinically overt (Lim, 2014). Some changes are present in the kidney function like raised kidney blood flow, hypertrophy of the renal and glomerular hyperfiltration. By good glycemic control, most of these changes could be inverted at an early stage. But they persevere in several patients and can be crucial in the subsequent evolution of clinical nephropathy (Mise et

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al., 2017). Normal human urine consists of very little amounts of albumin (Alb) only because less than 30 mg of Alb is excreted by healthful adults per 24 h. The glomerular filter dysfunction leads to inclusive leak for plasma proteins. Prevalent modification of podocyte foot operations is seen in different shapes of glomerulonephritis (Campion et al., 2017).

The evolution of DN was characterized by elevates Alb secretion rate and glomerular hyperfiltration. Exact quantification of the quantity of Alb in the urine has a pivotal clinical notion, for example, the excretion quantities of Alb more than 300 mg/24 h is called macroalbuminuria while the excretion quantities of Alb between 30 and 300 mg/24 h is called microalbuminuria (MAU) (Debbarma et al., 2015). Once overt nephropathy happens, the glomerular filtration rate (GFR) declines gradually over a period of many years at a speed (around 2-20 mL/min/year) which vary highly from person to another. End stage renal disease (ESRD) enhances in about 50% of type1 diabetes mellitus (T1DM) people with overt nephropathy for 10 years whereas in more than 75% within 20 years (Drosos et al., 2018).

Many of people with T2DM may have MAU and overt nephropathy quickly following their diabetes diagnosis because diabetes is indeed existing, perhaps for several years, before the diagnosis. About 20 to 40% of T2DM patients with MAU advance to overt nephropathy, but only around 20% of them will progress to ESRD in 20 years after overt nephropathy onset (Elnajjar et al., 2016). The normal history of DN in T2DM patients may lead to death after 6 years of the persistent proteinuria onset. The aggregate series is identical in T2DM patients, but doubts may present due to imprecise date of the diabetes onset (Fiseha and Tamir, 2016). The decrease is more changeable in T2DM patients and the rate of progress to ESRD can be, as low as, 20% through 20 years. Ameliorations in the nephropathy management have expanded the course of time from continual proteinuria to kidney failure. Considering the raised happening of both DM and DN, the detection of early DN is of fundamental importance to supply suitable therapy that prevents or slows evolution towards ESRD (Sekulic and Sekulic, 2015).

Biomarkers have a crucial role in the first detection of DN. The detection of early DN comprises numerous new biomarkers. These biomarkers span the period of normoalbuminuria that predates MAU but also the development of renal involvement during MAU and macroalbuminuria (Kim et al., 2014).

L-Carnitine (LC) is a co-factor needed for the transportation of long chain fatty acids in the mitochondria for production of energy in peripheral tissues. LC may play a pivotal role in prohibiting generation of free radical, protecting tissues from impairment by fixing the oxidized lipids of membrane and preventing fatty acids beta-oxidation damage in mitochondria (Giudetti et al., 2016).

Also, osteoprotegerin (OPG) is a secreted glycoprotein which regulates the bone resorption. It is generated as a monomer (60 kDa) and congregated as a homodimer in the cell and then secreted, fundamentally, as a di-sulfide connected homodimer in the circulation. OPG was specified as a cytokine and member of the tumor necrosis factor (TNF) receptor superfamily (Xia et al., 2015).

Furthermore, sialic acid (SA), a general expression for a family of acetylated derivatives of neuraminic acids, is a vital element of glycolipids and glycoproteins. It works as a cofactor for several cell receptors and associates positively with many of the serum acute phase reactants (Zhu et al., 2017).

Moreover, fibronectin (FN), a high molecular weight (440 kDa) glycoprotein, is a substantial part of the glomerular extracellular matrix (ECM). It is generated in the platelets, liver and vascular endothelia. It shows in a soluble format in plasma and on the cell surface in fibrillar format (Uwaezuoke, 2017).

Although the scientific community has started resolving the secrets of the close linkage between insulin resistance (IR), some blood and urine biomarkers, some trace elements (Selenium [Se], Zinc [Zn] and Magnesium [Mg]) and their physiological impacts, a lot is still remaining to be discovered. In Basrah governorate (southern of Iraq), to date, no study has been investigated on IR and its effects on some blood and urine biomarkers in DN. Therefore, the present study is aimed to assess the effect of IR on some blood and urine biomarkers as excellent indicators for early-stage DN in patients with T2DM.

MATERIALS AND METHODS

Subjects

Sixty-three subjects (men and women) suffering from T2DM were enrolled in this study and they were divided into two groups: the first group consisted of 31 patients suffering from DN (15 men and 16 women) while the second group consisted of 32 patients free from DN (15 men and 17 women). These subjects were matched with 33 healthy controls (16 men and 17 women). This study is a cross-sectional clinical study and it was conducted based on the Helsinki ethical guidelines. Patients with the following inclusion criteria were enrolled in this study; age between 36 and 65 years old; T2DM patients (with or without DN) were diagnosed by clinicians in the diabetes and endocrine glands centre in Al-Mawany teaching hospital. The control group was health individuals, not suffering from T2DM nor having any family history of T2DM, not suffering from any acute or chronic cardiovascular diseases and not taking any drug believed to alter plasma glucose level. All the volunteers had a stable clinical course for at least 3 months. Patients who were pregnant (women), had angina or heart failure, renal failure, hypertension, alcoholics, T1DM, urinary tract infection, uncontrolled thyroid disorders, severe liver dysfunction, human immunodeficiency viruses (HIV) infections, pancreatic diseases, hormonal abnormalities, genetic syndromes and severe concurrent illness were excluded from the study. Demographical data were collected via a structural interview that was conducted during the visit. Standard self-administered questionnaire paper is used to

define the age, health habits (exercise, smoking or alcohol consumption), duration of DM and DN, medical history and current medications. Diagnosis of T2DM patients was based on the recommendation of American Diabetes Association (ADA, 2018). Duration of DM was considered as the time from which the patient was diagnosed with T2DM. DN was diagnosed on the basis of GFR level [<60 mL/min/1.73 m²] (Jerums et al., 2012) or persistent MAU (>19 µg/mL) in the morning urine samples of patients (Zanetti et al., 2020).

Samples collection and preparation

All samples (blood and urine) were collected in the morning between 09:00 and 10:00 am after 12 h fasting time and 30 min of rest in the supine position. 10 mL of venous fasting blood sample was collected from each subject by vein puncture then divided into two parts: the first part was whole blood obtained by adding 1 mL of blood into tubes (with anticoagulant) and shook gently to be utilized for the determination of Se level. The second part (9 mL) was moved to plain tube (without anticoagulant) which admitted clotting for 20 min at room temperature. After the blood had clotted, it was moved into a centrifuge at 402 × *g* for 20 min to get the serum. In addition, 20 mL of urine was collected from each subject as well and centrifuged at 402 × *g* for 20 min to remove all suspended particles and cell derbies aliquoted, the clear supernatant was collected carefully. Whole blood, serum and urine samples, for each participant, were utilized immediately in the estimation of variables in this study while the rest was stored in deep freezing (-80°C) until another use (Al-Fartosy and Mohammed, 2017).

Routine laboratory tests

Routine lab tests included determination of glucose, urea and creatinine levels were determined by UV-Vis Spectrophotometer (UV-EMC-LAB, Duisburg, Germany) by using the following kits (Randox, County Antrim, UK, Cat. No.: GL364; Linear, Barcelona, Spain, Cat. No.: 1156015; Randox, County Antrim, UK, Cat. No.: CR 511/S), respectively (Sirivole and Eturi, 2017).

Assay of blood and urine biomarkers

Serum (insulin, LC, OPG and SA) and urine (Alb and FN) biomarkers were assayed by human insulin ELISA kits. Sandwich ELISA technique was applied, and the level of each biomarker was measured by a standard curve. Insulin was assayed by the following kit (Calbiotech, California, USA, Cat. No.: IS130D) (Febbraio, 2017). While LC was assayed by the following kit (BT-Lab, Shanghai, China, Cat. No.: E3426Hu) (Bae et al., 2015). Whereas OPG was assayed by the following kit (BT-Lab, Shanghai, China, Cat. No.: E1558Hu) (Bernardi et al., 2016). Where SA was assayed by the following kit (BT-Lab, Shanghai, China, Cat. No.: E1620Hu) (El-Sayed et al., 2018). On the other hand, Alb was assayed by the following kit (Creative Diagnostics, New York, USA, Cat. No.: DEIA2299) (Campion et al., 2017). Finally, FN was assayed by the following kit (BT-Lab, Shanghai, China, Cat. No.: E2002Hu) (Indriani et al., 2020).

Ethical approval

All enrolled subjects signed informed consent to participate. The study was approved by the ethics committee of College of Science, University of Basrah (No.: 7/54/1879) and conducted in accordance with the Declaration of Helsinki.

Estimation of trace elements

Serum Zn and Mg were determined by using GBC 933 Plus flame atomic absorption spectrometry "AAS" (GBC, Braeside, Australia). While, whole blood Se was determined by Flame Atomic Absorption Spectrometer with Homemade Hydride Generation System (Shimadzu, Tokyo, Japan) (Al-Fartosy et al., 2019).

Calculations of some clinical parameters

Body mass index (BMI) was calculated by the standard BMI equation (Al-Fartosy et al., 2020a):

$$BMI\ (kg/m^2) = weight\ (kg)/height\ (m^2).$$

while insulin resistance (IR) was calculated by the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) equation (Al-Fartosy et al., 2020b):

$$HOMA\text{-}IR = Fasting\ insulin\ (\mu IU/mL) \times Fasting\ glucose\ (mg/dL) / 405.$$

whereas glomerular filtration rate (GFR) was calculated by the Modification of Diet in Renal Disease Study (MDRD) equation (Chen et al., 2016):

$$GFR\ (mL/min/1.73\ m^2) = 186 \times Serum\ Cr^{-1.154} \times age^{-0.203} \times 1.212\ (if\ subject\ is\ black) \times 0.742\ (if\ subject\ is\ female).$$

where creatinine clearance (CrCl) was calculated by the Cockcroft-Gault equation (Chen et al., 2016):

$$CrCl\ (mL/min) = (140 - age) \times (weight, kg) \times (0.85\ if\ female) / (72 \times Serum\ Cr).$$

Statistical analysis

Results were analysed using the statistical package for the social sciences (SPSS) software (Version 21) and the values were expressed as mean ± standard deviation (SD). The values of $p > 0.05$ was considered statistically not significant, $p < 0.05$ was considered as significant differences and $p < 0.01$ for highly significant in comparison with the corresponding control value (Al-Fartosy et al., 2019).

RESULTS

The general characteristics of all subjects participated in the present study were given in Table 1.

Data obtained indicated that there was a non-significant change ($p>0.05$) in body mass index (BMI) level in T2DM patients with and without DN as compared to control group as shown in Table 2. In addition, compared with normal controls, the results indicated that T2DM patients with and without DN had high significantly ($p<0.01$) increased levels of serum biomarkers (glucose, insulin, HOMA-IR and SA) as mentioned in Table 2. Furthermore, patients with T2DM with DN had a high significant increase ($p<0.01$) and patients with T2DM without DN had a significant increase ($p<0.05$) in the levels of serum (urea, creatinine and OPG) and urine (Alb and FN)

Table 1. The demographic characteristics of the present study.

Characteristics		T2DM patients		Healthy control
		With DN	Without DN	
Total subjects number		31	32	33
Age (years) (mean ± SD)		57.2 ± 4.4	53.9 ± 4.7	51.7 ± 5.0
DM Duration (years) (mean ± SD)		13.5 ± 2.5	5.2 ± 1.6	-
DN Duration (years) (mean ± SD)		6.8 ± 0.6	-	-
Demographic Area	Urban	28	27	29
	Rural	3	5	4
Educational background	Learned	25	24	26
	Illiterate	6	8	7
Smoking habits	Positive	0	0	0
	Negative	31	32	33
Food habits	Vegetarian	5	6	8
	Non-vegetarian	26	26	25
Employment status	Employed	19	14	28
	Not Employed	12	18	5
Subjects' gender	Men	15	15	16
	Women	16	17	17

SD: Standard deviation, DM: Diabetes mellitus, DN: Diabetic nephropathy.

biomarkers, compared to healthy control as demonstrated in Table 2. On the other hand, our data reported that GFR, CrCl and LC levels were decreased significantly in T2DM patients ($p<0.01$ in patients with DN and $p<0.05$ in patients without DN) as illustrated in Table 2.

Moreover, levels of blood trace elements (Se, Zn and Mg) were high significantly ($p<0.01$) lower in T2DM patients with and without DN as compared to control group as seen in Table 3.

DISCUSSION

To the best of our knowledge, this is the first study climbed on the objective of assessing the effect of IR on some blood and urine biomarkers and some trace elements levels in T2DM patients with and without DN in Basrah governorate (southern of Iraq). In the current study, the data presented that all the patients and healthy control subjects were non-smokers. Also, most of the volunteers from both patients and healthy control were from urban, all of them acquired a well-education and they had a good work place, as shown in Table 1. The major variations between urban and rural regions are the differences in food habits, genetic, social, psychic, pollution, environments and others raising dramatically in urban areas (Al-Fartosy et al., 2020b).

Obesity (elevated BMI level) perhaps acts as a diabetogenic factor via elevating resistance to the action of insulin in those genetically predisposed to enhance T2DM. IR leads to higher plasma levels of insulin which bring about an increase in appetite. Consequently, people eat more and gain more weight. So, obesity could be one of the etiological factors in the development of T2DM, and mostly because of loss of early phase insulin secretion in response to glucose which happens relatively earlier in the development of T2DM (Park et al., 2018). This loss is critically crucial as the early blast of insulin secretion plays a substantial role in priming target tissues of insulin, especially the liver responsible for normal glucose homeostasis after food uptake and mealtime glucose deflection take place when this process was deteriorated (Boughton et al., 2017). Obesity is considered one of the modifiable cardiovascular risk factors that is far more predominant in those people with T2DM than in the general population. Moreover, obesity and physical inactivity are important independent risk factors for T2DM in middle aged men (Wang et al., 2016).

Insulin resistance (IR) can be known as a form of biological misinformation in the body in which the insulin hormone receptors on the cell membrane are not responding suitably to the insulin. Thus, the glucose in blood becomes unable to reach into cells which could lead to a hypoglycaemic reaction. This condition makes

Table 2. Levels of serum and urine biomarkers in men and women of healthy control and T2DM patients with and without DN.

Biomarker	Gender	T2DM Patients										Healthy Control
		With DN					Without DN					
		Mean ± SD	SE	Range	95 % CI		Mean ± SD	SE	Range	95 % CI		Mean ± SD
					Lower	Upper				Lower	Upper	
BMI (kg/m²)	Men	31.2 ± 1.6	0.4	28.7 - 34.1	26.6	35.9	30.3 ± 2.5	0.6	26.3 - 34.6	23.1	37.4	31.5 ± 0.2
	Women	30.1 ± 0.5	0.1	29.8 - 32.1	28.7	31.5	30.8 ± 0.3	0.1	29.6 - 31	29.8	31.9	30.0 ± 1.7
Glucose (mg/dL)	Men	172.7 ± 3.3**	0.8	162.0 - 180.0	163.4	182.0	168.6 ± 3.0**	0.8	158.0 - 174.0	160.0	177.1	104.2 ± 3.8
	Women	162.3 ± 2.3**	0.5	159.0-171.0	155.8	168.8	158.7 ± 2.1**	0.5	151.0-163.0	152.8	164.7	99.6 ± 4.5
Insulin (µU/mL)	Men	29.0 ± 2.7**	0.7	24.6-33.4	21.5	36.6	28.1 ± 2.3 **	0.6	24.3-32.0	21.5	34.7	11.5 ± 1.8
	Women	28.7 ± 1.1**	0.2	26.8 - 30.6	25.6	31.9	27.9 ± 1.8 **	0.4	24.6 - 31.1	22.7	33.2	10.7 ± 1.1
HOMA-IR	Men	12.4 ± 1.3**	0.3	9.9 - 14.9	8.7	16.0	11.7 ± 1.1**	0.2	9.5 - 13.8	8.6	14.8	2.9 ± 0.5
	Women	11.5 ± 0.5**	0.1	10.5 - 12.9	10.0	13.0	10.9 ± 0.8**	0.2	9.2 - 12.5	8.7	13.2	2.6 ± 0.4
Urea (mg/dL)	Men	59.3 ± 3.9**	1.0	52.8 - 65.7	48.2	70.3	36.7 ± 2.5*	0.6	32.5 - 40.9	29.5	43.8	29.6 ± 4.0
	Women	53.9 ± 1.8**	0.4	51.0 - 56.8	48.9	58.9	30.1 ± 2.1*	0.5	24.9 - 37.0	24.2	35.9	25.0 ± 2.2
Creatinine (mg/dL)	Men	1.7 ± 0.1**	0.1	1.3 - 1.9	1.2	2.3	1.1 ± 0.1*	0.1	1.0 - 1.3	0.9	1.3	0.9 ± 0.1
	Women	1.4 ± 0.1**	0.1	1.3 - 1.5	1.2	1.5	0.9 ± 0.1*	0.1	0.8 - 1.0	0.8	1.1	0.8 ± 0.1
GFR (mL/min/1.73 m²)	Men	43.2 ± 6.9**	1.7	36.8 – 58.0	24.0	62.4	70.8 ± 7.3*	1.8	60.0 - 83.8	50.5	91.1	91.1 ± 6.8
	Women	40.4 ± 2.1**	0.5	37.0 - 44.1	34.3	46.4	64.2 ± 4.9*	1.1	56.6 - 72.9	50.6	77.9	80.1 ± 7.2
CrCl (mL/min)	Men	66.5 ± 8.8**	2.2	58.0 – 85.0	42.0	91.0	96.0 ± 10.9*	2.8	81.0 - 113.0	65.7	126.3	130.3 ± 12.1
	Women	62.8 ± 5.3**	1.3	54.0 – 71.0	48.1	77.6	99.7 ± 9.7*	2.3	85.0 – 116.0	72.8	126.7	126.0 ± 8.0
LC (nmol/mL)	Men	29.0 ± 2.5**	0.6	21.9 - 36.0	21.8	36.1	37.2 ± 4.4*	1.1	30.0 - 44.5	24.8	49.7	47.4 ± 5.3
	Women	28.2 ± 4.9**	1.2	20.1 - 36.3	14.4	42.0	35.3 ± 2.1*	0.5	27.7 - 39.6	29.4	41.	44.9 ± 4.1
OPG (ng/mL)	Men	3.2 ± 0.1**	0.1	3.0 - 3.4	2.9	3.5	2.7 ± 0.1*	0.1	2.4 - 2.9	2.2	3.1	1.9 ± 0.3
	Women	3.1 ± 0.5**	0.1	2.3 - 4.0	1.7	4.6	2.6 ± 0.2*	0.1	1.9 - 3.3	1.9	3.2	1.7 ± 0.1
SA (mg/dL)	Men	87.6 ± 4.4**	1.1	80.4 - 94.9	75.2	100.1	79.4 ± 4.7**	1.2	71.6 - 87.2	66.1	92.7	61.0 ± 2.9
	Women	85.7 ± 2.8**	0.7	80.8 - 90.4	77.7	93.7	77.3 ± 2.6**	0.6	69.2 - 84.5	70.0	84.5	60.3 ± 5.9
Alb (µg/mL)	Men	29.5 ± 4.6**	1.1	22.0 - 37.0	16.7	42.4	11.1 ± 3.1*	0.8	6.1 - 16.2	2.5	19.8	6.1 ± 2.1
	Women	28.5 ± 5.2**	1.3	19.9 - 37.0	13.9	43.1	10.6 ± 2.5*	0.6	6.4 - 14.9	3.4	17.8	5.8 ± 2.0
FN (ng/mL)	Men	16.4 ± 4.4**	1.1	9.2 - 23.5	4.2	28.6	12.2 ± 3.4*	0.8	6.6 - 17.8	2.6	21.8	8.9 ± 3.1
	Women	15.3 ± 4.1**	1.0	10.0 - 24.1	4.0	26.7	11.3 ± 3.4*	0.8	5.2 - 17.0	1.8	20.8	8.0 ± 0.8

Data are presented as mean ± standard deviation (SD); SE: Standard error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence intervals (Lower and Upper); significance level is indicated by *, Where: $p > 0.05$: p-value not significant, * indicates $p < 0.05$ (p-value significant); ** indicates $p < 0.01$ (p-value high significant), in comparison with the corresponding control value. T2DM: Type2 Diabetes Mellitus, DN: Diabetic Nephropathy, BMI: body Mass Index, HOMA-IR: Homeostatic Model Assessment for Insulin Resistance, GFR: Glomerular Filtration Rate, CrCl: Creatinine Clearance, LC: L-Carnitine, OPG: Osteoprotegerin, SA: Sialic Acid, Alb: Albumin, FN: Fibronectin.

Table 3. Levels of blood trace elements in men and women of healthy control and T2DM patients with and without DN.

Biomarker	Gender	T2DM Patients										Healthy Control
		With DN					Without DN					
		Mean ± SD	SE	Range	95 % CI		Mean ± SD	SE	Range	95 % CI		Mean ± SD
					Lower	Upper				Lower	Upper	
Se (ng/mL)	Men	63.5 ± 3.4 **	0.8	62.1 - 67.6	54.1	73.0	70.9 ± 1.8**	0.4	69.3 - 73.6	65.7	76.0	92.8 ± 1.0
	Women	50.8 ± 1.9 **	0.4	49.2 - 55.0	45.4	56.2	59.5 ± 1.7**	0.4	58.4 - 64.2	54.7	64.3	80.1 ± 3.2
Zn (µg/mL)	Men	0.8 ± 0.2 **	0.1	0.5 - 1.3	0.1	1.5	0.9 ± 0.1**	0.1	0.5 - 1.2	0.5	1.4	1.5 ± 0.3
	Women	0.7 ± 0.2 **	0.1	0.4 - 1.1	0.1	1.3	0.7 ± 0.1**	0.1	0.5 - 1.1	0.2	1.2	1.0 ± 0.3
Mg (µg/mL)	Men	16.7 ± 1.3 **	0.3	14.4 - 18.9	12.8	20.5	19.9 ± 1.1**	0.2	18.1 - 21.7	16.8	23.0	23.4 ± 3.7
	Women	12.9 ± 2.5 **	0.6	8.7 - 16.9	5.9	19.9	14.9 ± 1.4**	0.3	12.4 - 17.3	10.9	18.9	20.1 ± 2.68

Data are presented as mean ± standard deviation (SD); SE: Standard error, Range: is the difference between the highest and lowest values in the set, 95% CI: Confidence intervals (Lower and Upper); significance level is indicated by *, Where: $p > 0.05$: p-value not significant, * indicates $p < 0.05$ (p-value significant); ** indicates $p < 0.01$ (p-value high significant), in comparison with the corresponding control value. T2DM: Type2 Diabetes Mellitus, DN: Diabetic Nephropathy, Se: Selenium, Zn: Zinc, Mg: Magnesium.

the pancreas produces high doses of insulin to endeavour getting the glucose out of blood into cells. So, this leads to decrease in the ability of insulin to adjust and signal changes in the levels of glucose in the blood and perhaps grows IR (Al-Fartosy et al., 2020b). Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was used as a substitute measure of IR in our study. Although HOMA-IR was not the gold standard for assessment of insulin sensitivity, but it was a clinically useful index in many studies. Pancreatic β-cells function may be damaged during adolescence or later. Its deterioration ranges from hyperinsulinemia, secondary to IR, with normal glucose tolerance to β-cells failure with T1DM. When IR is present, or when insulin secretion is decreased in the later stages of the disease, free fatty acids (FFAs) are released in large amounts followed by an elevated production of glucose, triglycerides (TGs) and very low-density lipoprotein (VLDL) (Al-Fartosy and Mohammed, 2017). In addition, FFAs also decreased insulin sensitivity in muscles by discouraging insulin mediated glucose uptake. On the other hand, elevated blood glucose level, and to some extent circulating FFA, raise insulin secretion and lead to increase hyperinsulinemia. It is obvious that IR causes blood glucose and FFAs levels to rise, thus, worsening the IR and hyperglycemia with released FFAs further rises insulin secretion forming a wicked circle (Cicero et al., 2015). Furthermore, the pivotal role of the fat mass does not rule out the significance of heritability in the evolution of the metabolic syndrome. Some environmental factors, such as the obesity epidemic due to the lack of physical exercise and increased caloric intake, are obviously responsible for the present elevation in the incidence of the metabolic syndrome worldwide (Febbraio, 2017). So far, the predilection to get weight is highly individual and determined by genetic factors. It has been speculated that TG's accumulation in skeletal muscles plays a lineal role in the aetiology of IR. The results of several studies have demonstrated that the degree of IR is positively correlated with intramuscular TG's content. So, chronic hyperglycemia and dyslipidemia in T2DM can both produce hurtful effects on β-cell structure and function (Xin et al., 2019). Even though inter-relationships between lipotoxicity and glucotoxicity have not yet been illustrated, it is supposed that glucotoxicity could lead to β-cell apoptosis independently of dyslipidemia. While lipotoxicity may damage β-cells only in the presence of hyperglycemia. In the situation of normoglycemia, raised FFAs must oxidized easily in the mitochondrion and should not damage the β-cell of pancreas. Moreover, as DN progress, IR may be stimulating the decrease in renal function toward ESRD. High TG's levels are a risk factor for proteinuria development and TG-rich apolipoprotein B-containing lipoproteins clearly promote the progression of renal insufficiency (Mahfouz et al., 2016).

Our data revealed that urea and creatinine levels

were increased while GFR and CrCl levels were decreased in T2DM patients with and without DN when compared with healthy controls, this agrees with another study with the same results (Chen et al., 2016).

L-carnitine (LC) is an essential co-factor for FA metabolism and other metabolic pathways, with body stores maintained primarily in skeletal muscle. The reduction in some catalysts and co-factors used by humans to biosynthesize LC from L-lysine and L-methionine via a series of reactions in the liver, kidney and brain such as S-adenosylmethionine, α-ketoglutarate, oxygen, ascorbic acid, iron, glycine, vitamin B6 and γ-butyrobetaine hydroxylase could lead to decrease in LC level in T2DM patients with and without DN (Giudetti et al., 2016). Moreover, the combination of impaired glucose tolerance (IGT) with simple obesity may stimulate the progress of hepatic ketogenosis in coupling with a raised SCAC (short-chain acylcarnitine) and an elevation in carnitine acylation with a decrease in LC levels (Bae et al., 2015).

Osteoprotegerin (OPG), a soluble glycoprotein composed of 380 amino acid residues, may be found in osteoblasts of the bone, vascular endothelial cells and smooth muscle cells. Obesity could increase the OPG level in T2DM patients with and without DN as OPG expression has been assured in adipose tissues as well. Abdominal adipose tissue is the largest fat tissue store in the body and correlates with metabolic syndrome, cardiovascular disease risk and other systemic inflammatory markers and may influence atherosclerosis. Adipose tissue releases many adipokines but also there is elevating evidence that there is a hormonal cross-link between adipose tissue and bone which leading to raise OPG level (Bernardi et al., 2016). In addition, insulin resistance (IR) may have a pivotal role in increasing OPG level in T2DM patients with and without DN because it could be the potential mechanism for elevating serum lipid levels especially levels of TG and this led to increase OPG levels. Moreover, increased creatinine and cystatin C levels may elevate the OPG level in T2DM patients with and without DN because cystatin C is a cysteine proteinase inhibitor that declines osteoclastogenesis by interfering at a late stage of pre-osteoclast differentiation which led to decreased protein clearance and decline in GFR as a probable cause of age-linked OPG elevation (Xia et al., 2015).

Sialic acid (SA) is a fundamental component of glycoproteins and glycolipids; it has a major role as a co-factor for receptors of many cells. Increased levels of SA in blood serum of T2DM with and without DN could be attributed to the fact that SA is a crucial acute phase reactant and maintains the negative charge of kidney glomerular basement membrane that is one of the master organizers of membrane permeability. Therefore, vascular endothelium holds higher levels of SA and hence comprehensive pathological states when there is tissue deterioration, tissue reproduction and inflammation connected with T2DM accounts for its sloughing to the circulation leading to raise vascular permeability and overall elevated SA level (El-Sayed et al., 2018). From the other hand, obesity, hyperglycemia and IR could develop inflammation and this may play a factor connecting DM to the development of diabetic complications. Increased glucose levels could enhance inflammation by raising oxidative stress. Another probability is that inflammatory response is a result of vascular complications after DM. Hence, diabetic vascular complications can lead to a severe tissue deterioration which may trigger excretion of large levels of local cytokines from cellular infiltrates like endothelial cells and macrophages. Moreover, this excretion of cytokines could stimulate an acute phase response with release of acute phase glycoproteins with SA from the liver tissue into the general circulation again which raised the levels of SA in blood (Zhu et al., 2017).

Selenium (Se), a trace element, is a major component in glutathione peroxidase (GPx) which is one of the main antioxidant enzymes in the human body and responsible for preventing the production of free radicals, decreasing their activity or destroying them (Al-Fartosy et al., 2020a). Se levels were found to be significantly lower in T2DM patients with and without DN when compared with healthy control group. It is known to work as an antioxidant and peroxynitrite scavenger when integrated into selenoproteins. This lack in Se levels may lead to oxidative stress, decline insulin secretion and elevated IR in some empirical models; thereby, perhaps taking a causal function in the forward and pathogenesis of T2DM. Moreover, elevated oxidative stress and glycosylation play a main pathogenic role in diabetic endothelial cell dysfunction in T2DM patients with and without DN (Al-Fartosy et al., 2019).

Zinc (Zn) is one of the fundamental trace elements which are involved in the synthesis, secretion, conformational integrity and storage of insulin. Our study reported that Zn levels in T2DM patients with and without DN were lower than the control group. The probable explanation of the current findings may come as the following reasons: in the mammalian pancreas, Zn is fundamental for the correct processing, secretion, storage and action of insulin in beta cells. Insulin is stored in secretory sacs or granules, where two Zn^{2+} ions coordinate six insulin monomers to generate the hexameric form on which maturated insulin crystals are based (Al-Fartosy et al., 2017a). It is also known that many other chronic disorders like DM could decrease Zn levels, for example, DM increases the excretion of minerals such as Zn in urine or may decrease gastrointestinal absorption of Zn. Also, hyperglycemia in DM is often linked with hyperzincuria, which is of kidney origin, and raise urinary loss of Zn^{2+} and declines of its level in entire body Zn^{2+} (Al-Fartosy et al., 2017b). Kidney tubular flaw in handling Zn and glucose-induced, osmotic diuresis are other probabilities. Furthermore, Zn may

enhance glycaemia and a restored Zn status in patients with T2DM (with or without DN) may oppose the harmful effects of oxidative stress which help to prevent complication beneficial antioxidant effects in people with T2DM. Zn has been illustrated to have particularly importance in the light of the mischievous outcomes of oxidative stress in subjects with DM. Zn has antioxidant properties, thus, it can balance macromolecules against radical stimulated oxidation (Al-Fartosy et al., 2020a).

Magnesium (Mg) is a trace element which is responsible for maintaining some body functions. Mg is demanded for several enzymes' activities and for neuromuscular transmission. In our current study, the serum level of Mg showed a high significant decreasing in T2DM patients with and without DN when compared with healthy subjects. Mg is essential for many enzymes that play a key role in glucose metabolism (Al-Fartosy et al., 2020b). The hypomagnesaemia in T2DM patients with and without DN in our study may be due to poor dietary intake, elevated urinary loss because of hyperglycemia, impaired absorption of Mg, imperfect Mg reabsorption from kidney tubules, osmotic diuresis and loss of plasma protein bound to Mg. Mg diminution is said to decrease the insulin sensitivity, thereby, raising the risk of secondary complications. Hyperglycemia leads to decline cellular Mg levels. Hypomagnesaemia leads to decreased function of Mg dependent enzymes, collagen and ADP-induced platelet convenience and oxidative stress. Moreover, insulin lack and resistance lead to decreased tubular reabsorption of Mg and resulted in hypomagnesemia which prefer the onset and development of diabetic microangiopathy via a decrease in activity of Na^+/K^+ ATPase pump. So, hypomagnesemia alone foretells the progression to ESRD in patients with advanced DN (Al-Fartosy et al., 2017a).

Fibronectin (FN), a protein with high molecular weight, is an extracellular matrix (ECM) protein and is congregated by cells into elastic and insoluble fibrils. The significant increase in FN level in T2DM patients with and without DN in our study might be due to hyperglycemia because it increases the mRNA levels of FN in the kidney cortex and leads to the overproduction of FN in kidney tissues. Hence, the glycemic disruption is a crucial factor that raises FN synthesis in the kidney, eventually leading to diabetic glomerular injury. As FN is produced by kidney mesangial cells, it was also found that FN in these cells is elevated proportionately in the disease states characterized by mesangial expansion including diabetic nodules (Lee and Choi, 2015). Furthermore, the significant elevation in FN level in T2DM patients with and without DN in our study could be due to connective tissue growth factor (CTGF) because it plays a key role in glomerular alteration in diabetic sclerosis because this mediator stimulates transient actin cytoskeleton disassembly in mesangial cells, high production of FN and mesangial cell hypertrophy (Indriani et al., 2020).

Additionally, protein kinase C (PKC) may play a pivotal role in raising FN levels in T2DM patients with and without DN through a complex mechanism involving its isoforms (PKC-α, PKC-β, and PKC-ε). These isoforms have been engaged as mediators of kidney fibrosis and mesangial expansion via upregulating of vascular endothelial growth factor (VEGF) expression in mesangial cells, as well as transforming growth factor-β (TGF-β) and FN in the glomeruli. Also, NADPH oxidase-driven renal oxidative stress stimulates mesangial expansion and albuminuria by elevating the expression of renal FN (Uwaezuoke, 2017).

Conclusion

The results of this study illustrate that obesity is a case accompanied by elevated levels of insulin and glucose which are the vigorous indicators for evaluation of the IR syndrome in diabetic patients especially with kidney disease. In addition, kidney disease is linked with the raising of IR and BMI levels in T2DM patients which had been observed via decreasing the levels of GFR, CrCl and increased serum urea and creatinine levels. Furthermore, LC had a renoprotection features through anti-inflammatory and anti-sclerotic effects as well as its ability to improve insulin sensitivity in insulin resistant diabetic patients. Therefore, decreased levels of serum LC may represent a powerful indicator for evaluation of the oxidative stress syndrome in diabetics than non-diabetics. Additionally, the increase in OPG levels is an independent risk factor for MAU and may be involved in vascular calcification independently of progression of DN in patients with T2DM. Further, elevated levels of serum SA are strongly associated with the presence of nephropathy and it could be representing a predictor of kidney dysfunction in DN. Besides, the decrease in some essential antioxidant trace elements levels, such as Se, Zn and Mg, is a powerful indicator for evaluation of the oxidative stress syndrome in diabetic patients with and without DN than non-diabetics. Moreover, urinary biomarkers like Alb and FN and the combination of these two biomarkers demonstrated an excellent diagnostic value for early-stage of DN in patients with T2DM. Finally, these investigated clinical biomarkers must be used in the future for early detection of DN in men and women of T2DM.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Correlation between glycated hemoglobin and venous blood sugar in diabetic patients monitored in Abidjan

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The aim of this study was to determine the correlation between glycated hemoglobin and blood sugar levels in diabetic subjects carried out in Abidjan. This cross-sectional study included 100 patients with diabetes monitored, for three months, for whom glycated blood glucose and hemoglobin were performed, this after informed consent of the patients. Pearson and Spearman correlation tests were used, at the 5% threshold. The patients with normal HbA1C and normal blood glucose accounted for 55.34 and 32%, respectively. A sedentary lifestyle and body mass index > 25 kg/m² were associated with a significant increase in the risk of increased blood glucose and HbA1C. The presence of a complication was associated with a 3.06-fold higher risk of high glycated hemoglobin (p= 0.0073), while blood glucose was not significantly associated with the onset of complications. Glycated hemoglobin was significantly correlated with blood glucose with a correlation coefficient of 0.4412 (p 0.0001). In multivariate analysis, hyperglycemia was significantly associated with alcohol consumption and non-compliance with antidiabetic treatment. Poor compliance was significantly associated with increased glycated hemoglobin (>7%) and tobacco consumption. The glycated hemoglobin was highly correlated with blood sugar, and was found to be a better predictor of diabetes complications than glycemia.

Key words: Diabetes mellitus, glycated hemoglobin, blood sugar, Abidjan, Côte d'Ivoire.

INTRODUCTION

The complications of diabetes are influenced not only by the duration of diabetes but also by the average level of chronic glycemia which is measured most reliably with glycated hemoglobin (HbA1C) assay (Lenters-Westra and Slingerland, 2008; ADA, 2009). The most common type of diabetes is type 2, long-asymptomatic, which

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usually occurs after a long period of insulin resistance and metabolic disorders (Colette and Monnier, 2010).

In 2010, in sub-Saharan Africa, more than 12 million people were living with diabetes and 330,000 people died from related complications (ADA, 2009, 2011). In diabetes, dietary measures, most of which are associated with drug treatments, are essential for a good patient balance (Colette and Monnier, 2010). The aim of these measures is to reduce insulin resistance, glycemic, lipid and blood pressure disturbances, and to prevent the disease from worsening (Verma et al., 2006; Colette and Monnier, 2010). The simple determination of blood glucose levels on a periodic basis at the time of checkups prior to visits to the medical practitioner cannot provide a correct picture of the long-term control of diabetic disease. To overcome this deficit, biochemical markers, including HbA1C, are used to help assess the glycemic balance of patients (Shubrook, 2010).

The HbA1C assay is used to estimate the mean level of blood sugar in the two months preceding the baseline (Kilpatrick, 2008). The study of HbA1C and blood glucose was carried out in numerous studies in both Caucasian and Black subjects (Kilpatrick, 2008; Shubrook, 2010; Ercan, 2014; Zendjabil, 2015). Thus, Gillery (2013) in France and Selvin et al. (2010) in the United States, made it possible to study cardiovascular risk in diabetic subjects and even to propose new diagnostic methods for diabetes. In North Africa, Zendjabil (2015) assessed cardiovascular risk in diabetic patients using the HbA1C assay. However, very few studies on HbA1C and glycaemia were carried out in Côte d'Ivoire. The objective of this work was to study the correlation between HbA1C and glycemia in diabetic subjects in a private health facility in the municipality of Yopougon.

MATERIALS AND METHODS

This is a descriptive and analytical cross-sectional study involving 100 patients, recruited for diabetology consultation at a private health facility in Yopougon (Abidjan), which lasted 3 months. Patients with diabetes, regardless of gender, with an age ≥18 years, who accepted the sample and gave informed consent, were selected for blood glucose and HbA1C. Patients with other associated conditions such as high blood pressure were not selected. A questionnaire was used to study certain epidemiological and clinical parameters such as, sedentary behaviour, body mass index (BMI), smoking and alcohol consumption, regularity of treatment follow-up, type of treatment, the quality of adherence to treatment, and the occurrence or non-occurrence of complications. Measurements of weight and height were made according to the WHO (2006) standard, in indoor garment, without footwear. The weight was obtained using a mechanical person's scale (make "SEVERIN PW 7010-Tasteful Technology"), with an accuracy of 0.1 kg.

Height was measured using a height gauge (make "SECA 206") with an accuracy of 0.1 cm. Overweight and obesity were determined by calculating the BMI [BMI (kg/m^2) = Weight/Size 2], which are defined respectively by BMI > 25 and 30 kg/m^2. The samples were taken from people with diabetes on an empty stomach the day before. The blood was collected in two tubes: one containing both an anti-glycolytic and an anticoagulant, and another containing ethylene diamine tetra-acetic acid (EDTA). Each tube contained 5 mL of blood collected. Glycated haemoglobin was determined by immunoturbidimetry method and blood glucose, by classical glucose-oxidase-peroxidase enzymatic method on a Cobas E 400 automaton. The reference values for HbA1C were 6.5% and for fasting blood glucose between 0.60 and 1.10 g/L (ADA, 2011). The samples were taken with informed consent from the patients. This study was approved by the Ivoirian Ethics Committee for Clinical Research (Approval number: 141/MSHP/CNER/01/2017). The analysis of the variables was done by the Pearson and Spearman correlation tests, with a significance threshold of 5%.

RESULTS

The sex ratio was 1.17 (1.17 men for a woman; that is, 54 men for 46 women). The proportion of type 2 diabetics was the highest at 96% and that of type 1 diabetic was weak at 4%.

Overweight patients accounted for 33% and obese subjects 41% of patients. The proportion of overweight and obese subjects was therefore 74%. Sedentary lifestyle was one of the most important risk factors and was observed in 59% of patients. Alcohol consumption was observed in 44% of patients and we recorded 19% of patients consuming tobacco. The healthy diet was part of the treatment of almost all patients (99% of cases). The treatment was regularly followed in 94% of the diabetic patients.

Table 1 shows that patients had an average age of 49.2±10.96 years with extremes of 13 and 82 years. The high prevalence of age was between 40 and 50 years (81.58%).

Table 2 shows the distribution of blood glucose and HbA1C by presence of complications. Blood glucose was not significantly associated with the occurrence of complications. The presence of a complication was associated with a 3.06 times higher risk of high HbA1C (Table 2).

The correlation curve between HbA1C and glycemia is as shown in Figure 1. HbA1C was significantly correlated with blood glucose. The correlation coefficient r was 0.4412 and a p0.0001.

Table 3 shows the distribution of blood glucose and HbA1C by BMI and lifestyle. A sedentary lifestyle and BMI > 25 kg/m^2 were associated with a significant increase in the risk of increased blood glucose and HbA1C.

Table 4 shows the distribution of blood glucose and HbA1C by treatment adherence. Poor adherence to treatment was associated with an increased risk of high mean blood glucose and high HbA1C.

Table 5 shows analysis of hyperglycemia and HbA1C with BMI, lifestyle, treatment and complications. Hyperglycemia and HbA1C were significantly associated with poor adherence to antidiabetic treatment. The type of treatment and the degree of obesity did not influence

Table 1. Variation in glycemia and glycated hemoglobin by age group.

Age (years)	High blood sugar (>1.1 g/L)			HbA1C High (>6.5%)		
	n (%)	p-value	OR	n (%)	p-value	OR
≤30	2/4 (50)	0.1022	1 (Ref)	3/4 (75)	0.2043	1 (Ref)
30-40	9/16 (56.25)		1.30	10/16 (62.50)		0.56
40-50	31/38 (81.58)		4.43	14/38 (36.84)		0.19
50-60	18/32 (56.25)		1.29	11/32 (34.38)		0.17
>60	8/10 (80)		4.00	5/10 (50)		0.33

HbA1C, Glycated haemoglobin; p-value (≤ 0.05) is statistically significant; n, number of patients; OR, odds ratio; Ref, Reference group.

Table 2. Distribution of glycemia and glycated hemoglobin level according to the presence of complications.

Presence of complications	High blood sugar (>1.1 g/L)			HbA1C High (>7%)		
	n (%)	p	OR	n (%)	p	OR
Yes	36/52 (69.23)	0.7836	1.13	29/52 (55.77)	0.0073	3.06
No	32/48 (66.67)			14/48 (29.17)		

HbA1C, Glycated haemoglobin; **n**, number of patients; p-value (≤ 0.05) is statistically significant; OR**,** odds ratio.

Table 3. Glycemia and HbA1C distribution by BMI and lifestyle.

Variable	Glycemia			HbA1C		
	n (%)	p	OR	n (%)	p	OR
Sedentary lifestyle	44/59 (74.58)	0.0308	2.08	27/59 (45.76)	0.0032	1.32
Tobacco	11/19 (57.89)	0.2941	0.58	12/19 (63.16)	0.0486	2.76
Alcohol	33/44 (75)	0.1835	1.80	21/44 (47.73)	0.3973	1.41
BMI > 25 kg/m²	50/74 (67.57)	0.0157	2.93	36/74 (48.65)	0.0442	2.57

HbA1C, Glycated haemoglobin; n, number of patients; p-value (≤ 0.05) is statistically significant; OR, odds ratio; BMI, Body Mass Index.

Table 4. Glycemia and glycated hemoglobin distribution by treatment adherence.

Adherence	Glycemia			HbA1C		
	n (%)	p	OR	n (%)	p	OR
Poor	39/48 (81.25)	0.0064	3.44	36/48 (75)	<0.0001	19.29
Good	29/52 (55.77)			7/52 (13.46)		

HbA1C, Glycated haemoglobin; n, number of patients; p-value (≤ 0.05) is statistically significant; OR, odds ratio.

changes in glycated blood glucose and hemoglobin.

DISCUSSION

Variation by age group

The present study is the first concerning correlation between HbA1C and glycemia in Abidjan. The age of most patients was between 40 and 50 years old, with an average of 49.2±10.96 years and extremes of 13 and 82 years (Table 1), and a sex ratio of 1.17. This distribution was comparable to that described by Qvist et al. (2008) which reported the mean age for all participants was 45.5. Mohammed et al. (2018) also showed that most affected age group by diabetes is between 45 and 55

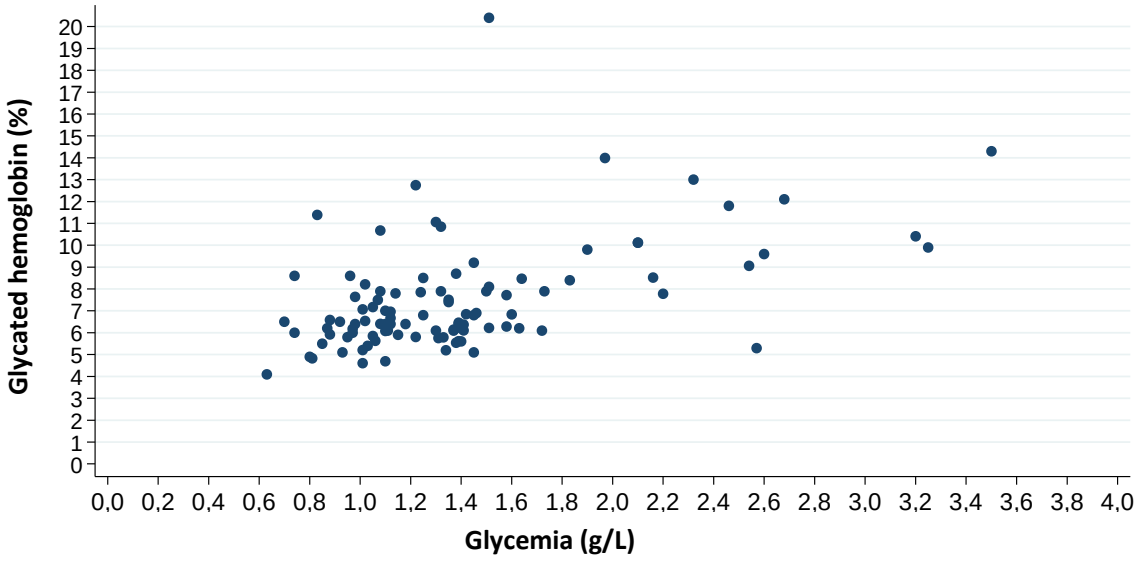

Figure 1. Correlation curve between glycated hemoglobin and blood glucose.

Table 5. Multivariate analysis of hyperglycemia and glycated hemoglobin with BMI, lifestyle, treatment and complications.

Variable		Glycemia		HbA1C	
		p	OR (IC 95%)	p	OR (IC 95%)
BMI	Lean		1 (Ref)		1 (Ref)
	Normal	0.471	4.43 (0.08-252.89)	0.517	5.09 (0.04-696.91)
	Overweight	0.625	2.71 (0.05-146.56)	0.225	20.85 (0.15-2824.44)
	Obesity 1	0.558	3.31 (0.06-182.02)	0.451	6.55 (0.05-870.65)
	Obesity 2	0.311	10.64 (0.11-1029.68)	0.332	13.36 (0.07-2508.33)
	Obesity 3	0.729	2.11 (0.03-146.51)	0.760	2.22 (0.01-377.56)
Sedentary lifestyle		0.698	1.28 (0.36-4.55)	0.438	1.90 (0.37-9.68)
Tobacco		0.260	0.366 (0.063-2.11)	0.019	17.60 (1.62-191.55)
Alcohol		0.016	5.25 (1.37-20.23)	0.163	2.99 (0.64-13.90)
Irregularity of the follow-up		0.065	0.10 (0.01-1.15)	0.210	0.10 (0.00-3.59)
Insulintherapy		0.323	3.43 (0.30-39.59)	0.403	0.24 (0.01-6.94)
ADO		0.250	4.17 (0.37-47.34)	0.777	0.61 (0.02-17.92)
Poor adherence		0.008	5.79 (1.58-21.16)	<0.001	103.02 (14.28-743.47)

HbA1C, Glycated haemoglobin; n, number of patients; p-value (≤ 0.05) is statistically significant; OR, odds ratio; Ref, reference group; BMI, body mass index; IC, confidence interval; ADO, oral antidiabetics.

years in a Tlemcen population in Algeria. The high proportion of subjects aged 40 to 50 justifies the recommendation of screening in subjects aged 45 and over (CDA, 2008). Our results showed that glycemia and HbA1C were not age-dependant. Several studies, including that of Verma et al. (2006) and Bouzid et al. (2011) previously reported in diabetic subjects that age was not associated with variation in HbA1C level, duration of diabetes, fasting blood glucose, glycated protein or glycated albumin. However, young age was cited as a factor associated with poor glycemic follow-up (Qvist et al., 2008), as well as old age (Wahba and Chang, 2007). According to the results of many longitudinal and cross-sectional studies, it has been demonstrated that the earliest detectable abnormality in non-insulin dependent diabetes mellitus (NIDDM) is impairment in the body's ability to respond to insulin (Lenters-Westra and Slingerland, 2008).

Occurrence of complications

Glycation of proteins affords an index for glycemic control, and moreover, literatures reported that increased

glycated proteins are linked to complications like nephropathy, cardiovascular diseases, and retinopathy among others (Megerssa and Tesfaye, 2015). In our study, glycated hemoglobin was found to be a good test for predicting complications (OR=3.06; p= 0.0073) (Table 2) and was reinforced by other work. Nakagami et al. (2017) have shown the risks of incident retinopathy and diabetic retinopathy were positively, continuously, and independently associated with HbA1C and fasting plasma glucose (FPG) levels in their cohort of Japanese participants without a previous history of diabetes. Furthermore, the adjusted hazard ratio for retinopathy were significantly higher in participants with an HbA1C level ≥6.5% or FPG level ≥7.0 mmol/L at baseline, relative to participants in the lowest categories of these variables (Nakagami et al., 2017). Also, Stratton et al. (2000) in the United Kingdom Prospective Diabetes Study Group (UKPDS) work on type 2 diabetes showed that the risk of myocardial infarction and microvascular complications decreased by 14 and 37%, respectively for any 1% decrease in HbA1C. Thus, the reduction of Hba1c, in the long term, led to a decrease in the risk of complications. Glycemia, on the other hand, showed no significant variation depending on whether or not complications occurred.

HbA1C and glycemia correlation

In the ADAG (HbA1C -Derived Average Glucose) study (Klonoff and Aimbe, 2014), conducted in type 1 and 2 diabetics and non-diabetic volunteers, the correlation between HbA1c and glycemia levels was higher (R 2 = 0.84) than the one found (R^2 = 0.44) (Figure 1). Likewise, Mohammed et al. (2018) showed a moderate positive correlation (r = 0.68) between the values of HbA1C and blood glucose in diabetic patients. This could be due to the fact that we performed a single dose of HbA1C and glycemia per patient, while in the ADAG cohort study, glycemia was measured before and after each meal and at bedtime.

This correlation seemed linear for all glycemia values. According to Bouzid et al. (2011) HbA1C values are correlated with fasting glycemia values and a 1% increase in HbA1c would approximate an increase of 18 mg/dl for fasting blood glucose found from the equation of the regression curve. Through irreversible formation and accumulation in red blood cells over 120 days of life, HbA1C accurately reflects changes in the glycemic balance over the previous three months. For example, glycated hemoglobin is the best diagnostic and follow-up parameter for diabetic disease, as it provides accurate information on long-term changes in glycemic balance, while venous and capillary glycemia measure the glycemic balance used in the short term and in emergency situations (Zafar et al., 2019). Thus, the correlation HbA1C/blood glucose level allows better control of glycemia (Mohammed et al., 2018).

Blood glycemia and HbA1C distribution by BMI and lifestyle

The vast majority of patients were physically inactive and had a higher than normal BMI, very often obese (Table 3). Our results showed that lack of physical activity increased the risk of obesity, hyperglycemia and HbA1C by more than 7% (Table 3). According to Rey-Lopez et al. (2008), a sedentary lifestyle is a factor of obesity. Physical inactivity and overweight may be responsible for significant changes in blood glucose and HbA1C. Studies showed that physical activity is significantly associated with a reduction in the risk of type 2 diabetes and that type 2 diabetes gradually settles in obese subjects (BMI 30) over the years (Gill and Cooper, 2008; Rey-Lopez et al., 2008).

It would be useful to advise diabetic patients to have a better quality of life compliance with lifestyle factors and diabetes self-management (Bennich et al., 2019). Thus, the practice of a regular physical activity, when combined with a hygienic-dietetic diet, would be effective in combating obesity, diabetes and its complications (Rey-Lopez et al., 2008; Adam et al., 2018; Seuring et al., 2019).

Multivariate analysis

Poor adherence to treatment was associated with an increased risk of high mean blood glucose and high HbA1C (Table 4).

Tobacco use was an independent predictor of increased HbA1C. Alcohol consumption was associated with a significant increase in the risk of hyperglycemia (Table 5). The relationship between alcohol consumption and insulin resistance results in a U-shaped curve. Another hypothesis is that smoking could influence the formation of HbA1c indirectly, independent of its effect resulting in elevated HbA1c levels (Jae et al., 2015). Our results could be attributed to alcohol consumption, a lifestyle parameter as important as smoking, was independently and negatively correlated with HbA1c levels, consistent with previous reports. Previous studies suggested that moderate alcohol intake might have protective effects on glucose metabolism by lowering insulin resistance (Greenfield et al., 2004; Bonnet et al., 2012). We recorded 48% of patients with poor adherence to treatment. The risk of hyperglycemia was 3.44 times higher with treatment non-compliance. Poor adherence to treatment was associated with a higher risk of high glycated hemoglobin (>7%). Poor adherence to treatment was also an independent predictor of hyperglycemia and an increase in HbA1C. The risk was even higher for glycated hemoglobin compared to blood glucose (103.02

vs. 5.79). Our results were consistent with those of Kumar et al. (2010) which reported that HbA1C had better specificity but less sensitivity to glycemia. This would justify some authors advocating reducing HbA1C to 6.1% instead of 6.5%. We can suggest as Razi et al. (2018) the necessity of determining the HbA1C cutoffs for detecting diabetes or prediabetes in the population, likewise the combination of these HbA1C cutoffs with fasting glycemia levels are required to determine diabetes and prediabetes more accurately, in a preventive purpose. Apart from diabetes, glycated hemoglobin has also proven to be a real marker of cardiovascular risk, since it has been associated with determination of serum sialic acid as risk factors in patients with myocardial infarction, who are non-diabetic (Basha and Kasi, 2019).

Conclusion

Our study showed that HbA1C was highly correlated with glycemia. Moreover, HbA1C was shown to be a better predictor of diabetes complications than glycemia. Thus, we recommend that the measurement of HbA1C should be integrated in health check of any person aged 40 or over, likewise organize awareness campaigns, screening and information of populations on metabolic diseases, especially diabetes. Future studies are needed with a larger sample of subjects to allow the use of this marker in the prevention of diabetes complications.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Evaluation of acute oral toxicity, hemato-biochemical activity and physiological responses of rabbits and rats administered *Moringa oleifera* leaf extract and meal

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The present study aims to evaluate acute oral toxicity of leaf extract of *Moringa oleifera* and determine the effect of the leaf's meals of this plant on hematological and biochemical parameters of the rabbit. Ten rats weighing 150 ± 200 g were used for the oral acute toxicity study. Five rats received orally a single dose of 2000 mg/kg of weight of aqueous extract and 5 rats used as control. Forty-eight New Zealand rabbits, weighing 968±100 g were randomly spread into 4 treatments groups containing 12 growing rabbits each. Rabbits are fed rations T_0, T_1, T_2 and T_3 containing respectively 0, 5, 10 and 15% of the leaf powder *M. oleifera* for 56 days. The results show that *M. oleifera* is not toxic at 2000 mg/kg. The results of the biochemical and hematological parameters obtained in the rabbits do not show any significant difference ($p > 0.05$) between the treatments. These results suggest that feeding *M. oleifera* up to 15% inclusion in rabbit diet will not have a deleterious effect on the health of rabbits. However, histological studies of the liver and kidney would be necessary to confirm the innocuousness of the plant.

Key words: *Moringa oleifera*, rats, rabbits, oral toxicity, hematological-biochemical parameters.

INTRODUCTION

Rabbit breeding is one of the most accessible breeding for most of the rural and peri-urban population because of the many benefits it offers (Akouango et al., 2014). Indeed, the domestic rabbit (*Oryctolagus cuniculus*) is an important source of good quality meat production for humans (Ahemen et al., 2013). Its consumption allows, among other things, a significant intake of proteins and essential amino acids such as lysine, leucine and arginine (Combes, 2004). The rabbit has a good lipid profile. Its tender and tasty meat is indeed an appreciable

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source of vitamins B3, B12, phosphorus and selenium (Ahemen et al., 2013; Combes, 2004). A white meat is recommended for individuals with diabetes, hypertension, etc.

In the light of all qualities that abound in this meat, it has an undeniable place as part of a healthy diet. As a result, the acceptability of this animal's meat is not a problem in the countries of the world.

In Benin, 64% of the population consumes farm rabbit meat at least once, and almost all (95% of consumers) appreciated it (Kpodekon et al., 2015). As a result, rabbit breeding is becoming a growing activity in Benin. This activity allows breeders to have a subsidiary income and provide animal protein of good quality for families. However, one of the factors hindering this breeding is feeding, which causes cases of various nutritional pathologies, the elongation of reproduction cycles, very long unproductive periods (Akouango et al., 2014), as well as mortality (Kpodekon et al., 2015). These pathologies of digestive origin have a detrimental effect causing rapid decline in production of meat of rabbit. Food-related expenses account for 70% of production costs. To overcome these problems, the use of unconventional local foods in the diet of these animals appears as a reasonable alternative (Aboh et al., 2002). Recently, the use of *Moringa oleifera* leaves as a source of cheaper protein in livestock feeding is becoming common place (Sarwatt et al., 2002). Indeed, this plant is rich in carotene, ascorbic acid, iron, methionine and cysteine. In addition, the leaves of this plant are energizing, rich in vitamins, and have the ability to strengthen the immune system (Ologhobo et al., 2014). They also cure diseases related to malnutrition, on the one hand, and on the other hand, diseases related to body mass and improve some blood parameters in rabbits (Osman et al., 2012). Indeed, the analysis of blood parameters is a means of establishing the state of health of an animal and thus determining the effect of the ingestion of food components on the blood composition (Church et al., 1984). Blood testing can therefore be considered as an appropriate measure of long-term nutritional status.

This study therefore aims to study the effect of the gradual incorporation of *M. oleifera* leaf meal on the blood parameters of domestic rabbits (*O. cuniculus*) in southern Benin; and also, to determine toxicological effects in rats.

MATERIALS AND METHODS

Plant source and processing

M. oleifera leaves were collected from Abomey-Calavi (Benin) city starting from month October to November 2016, and were certified at the National Herbarium of Benin under the reference AA66/1645/HNB. The plant material was air dried at room temperature in the laboratory for 10 days at a temperature of 24°C. The leaves were then milled with a grinder.

Extraction of plant

Cold extraction was done with water for 72 h at room temperature with intermittent shaking. A rotary evaporator set at 50°C was used to concentrate the extract. The dry extract obtained was kept in a refrigerator for later use. The plant was used in its powdered or meal form to determine its biochemical and hematological parameters on rabbits.

Experimental animals

Mice

Ten healthy female Wistar strain albino rats aged between 8 and 12 weeks, weighing 150 and 200 g were used for the toxicological study. The animals used were nulliparous and non-pregnant.

Rabbits

Forty-eight rabbits of New Zealand breeds weighing 968±100 g were used in this study. The animals were between 49 and 56 days old. The rabbits were randomly allocated to four (4) treatment groups with twelve (12) rabbits per treatment. The experimental groups were arranged as follows: group 1 (T_0) feeds with a ration containing 0% of *M. oleifera* leaf meal (control ration); group 2 (T_5) feeds with a ration containing 5% of *M. oleifera* leaf meal; group 3 (T_{10}) feeds with a ration containing 10% of *M. oleifera* leaf meal and group 4 (T_{15}): feeds with a ration containing 15% of *M. oleifera* leaf meal. Each group was then replicated thrice with 4 rabbits per replicate. The experimental feed and water were supplied *ad-libitum* twice daily at 7.00 and 16.00 h and the experiment lasted for 8 weeks.

Acute toxicity studies

This assay was done in accordance with the Organization for Economic Cooperation and Development (OECD, 2001) guideline. Five female and non-gravid Wistar rats weighing 150 to 200 g and aged between 8 and 12 weeks received by gavage, 2000 mg/kg aqueous extract of *M. oleifera* after being kept on fasting the previous night, while another four female rats (control) received water. The animals are observed closely during the first 4 h and daily for 14 days in order to monitor the weight changes, tremors, convulsions, salivation, diarrhea, lethargy, sleep, coma, death, and also changes in the skin, fur, eyes and behavioural pattern such as ingestive and physical (grooming, locomotion, inactivity). At the end of 14 days, hematological and biochemical parameters were measured.

Collection of blood and serum samples

Blood was taken at the end of 14 days. To sample blood, the rats were briefly anesthetized with isoflurane/oxygen and blood collected from the retro-orbital plexus according to Lenarczyk et al. (2013) in the heparinezed and dry tubes.

The blood in the heparinized tubes was used for haematological examination and the blood in the dry tubes was used for biochemical analysis on the same day.

Hematological and biochemical analyses of blood of rat

The hematological examinations were made using blood samples collected from retro-orbital of the experimental rats and conserved

Table 1. Ingredients used in the experimental diets in rabbits.

Ingredient	T_0 (0% *M. oleifera*)	T_1 (5% *M. oleifera*)	T_2 (10% *M. oleifera*)	T_3 (15% *M. oleifera*)
Maize (%)	14	14	14	14
Wheat bran (%)	20	20	20	20
Cotton waste cake (%)	7	7	7	7
Soya bean meal flour (%)	4	4	4	4
Corn bran (%)	14	14	14	14
Palm karnel cake (%)	40	40	40	40
Lysine (%)	0.1	0.1	0.1	0.1
Methionine (%)	0.1	0.1	0.1	0.1
Shells for oysters (%)	0.6	0.6	0.6	0.6
Phosphate (%)	0.2	0.2	0.2	0.2
Moringa oleifera leaf meal (%)	0	05	10	15

in capillary tubes (EDTA). These bloods were used to determine the Red Blood Cell (RBC), White Blood Cell (WBC), haemoglobin (Hb), and Packed Cell Volume (PCV) according to Duncan et al. (1994) methods. Blood constants such as Mean Corpuscular Haemoglobin Concentration (MCH), Mean Corpuscular Volume (MCV), and Mean Corpuscular Haemoglobin Concentration (MCHC) are calculated according to Ewuola and Egbunike (2008) methods. The serum used for biochemistry analyses such as Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Creatinine and Urea, were also measured.

Effect of *M. oleifera* leaf meal on hematological and biochemical parameters of rabbits

The meal that was obtained by grinding *M. oleifera* leaf was stored in airtight container until when needed for compounding or rather manufacturing. Four experimental diets comprising *M. oleifera* leaf meal has been used as a dietary supplement (Table 1).

T_0: received 0% of *M. oleifera* leaf meal
T_5: received 5% of *M. oleifera* leaf meal
T_{10}: received 10% of *M. oleifera* leaf meal
T_{15}: received 15% of *M. oleifera* leaf meal

Blood collection and evaluation of blood parameters on rabbits

At the end of the feeding period, blood samples were collected from the ear vein of each rabbit in the various groups, using a sterilized disposable syringe and needle. 2 ml blood was collected into labelled sterile vacuum tube containing ethylene-diamine-tetra-acetic acid (EDTA) as anticoagulant, of which another 3 ml of blood was collected into labelled sterile sample bottles without anticoagulant according to Ewuola et al. (2012).

The blood samples collected in EDTA was used for the determination of haematological parameters such as the RBC, WBC, Hb, and PCV as describe in Ewuola and Egbunike (2008). Blood constants such as MCH, MCV, MCHC and White blood differential counts (that is, Lymphocytes, Monocytes and Granulocytes) were determined to use appropriate formulae as described by Jain (1983). Biochemistry parameters such as glucose, urea, creatinine, cholesterol, total serum protein, serum albumin, Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and Alkaline phosphatase (ALP) were determined.

Statistical analysis

Analysis of variance (ANOVA) was done on the data collected. Duncan Multiple Range Test (Duncan, 1955) at a significant level of 0.05 was carried out in comparisons among dietary means. All computation was performed using statistical package SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Toxicological study

At the end of the two weeks of the experimental period (Day 14), no adverse effects were observed such as seizures, agitation, diarrhea, tremors, breathing difficulties and weight loss. Similarly, no mortality was recorded in animals throughout the experimental period as a consequent effect of administration of the *M. oleifera* leaf meal aqueous extract at a single dose of 2000 mg/kg body weight. This result shows that the dose of 2000 mg of the aqueous extract of *M. oleifera* leaves is lower than the LD50.

Figure 1 shows the weight variation of the rats tested as the controls throughout this test. There was an increase in the weight of the rats that received aqueous extract of *M. oleifera* leaf meal at a dose of 2000 mg/kg body weight.

Determination of hematological and biochemical parameters of rats

After 14 days of follow-up, blood samples were taken from the rats that received the extract as well as from the control rats for blood tests (Table 2). From this table it appears that no significant difference was noted on the two batches of rats, on both the globular constants and the blood cells.

Table 3 shows that the mean values of urea and

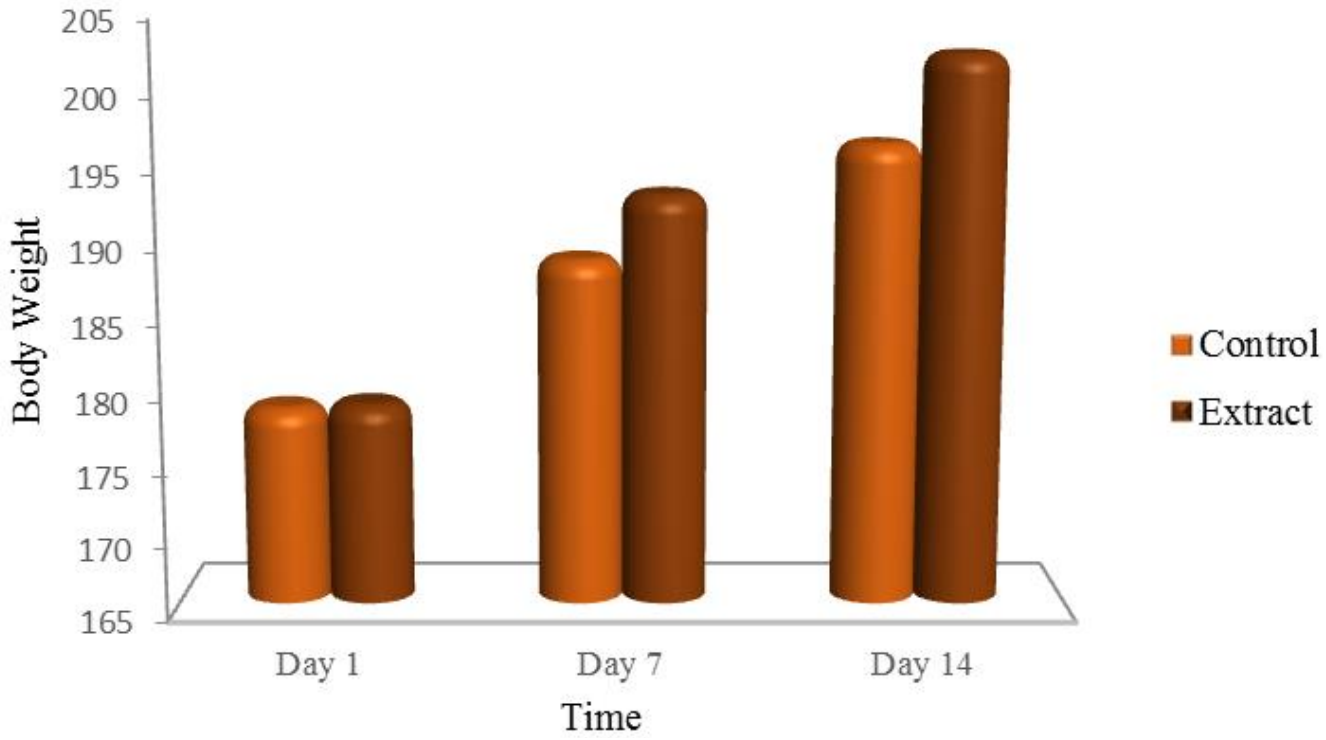

Figure 1. Changes in body weight of rats during the acute oral toxicity test.

Table 2. Effect of *Moringa oleifera* extract on haematological parameters in rats.

Parameter	Extract	Control
Red Blood Cell ($\times 10^6/mm^3$)	6.89 ± 0.45	6.16 ± 0.91
Haemoglobin (g/dl)	12.83±0.45	11.7±1.21
Packed Cell Volume (%)	38.36±1.20	33.43±4.80
Mean corpuscular volume (fl)	55.73±1.87	54.3±1.55
Mean Corpuscular Haemoglobin (pg)	18.56±0.68	19.03±1.00
Mean Corpuscular Haemoglobin Concentration (g/dl)	33.4±0.1	35.1±1.55
Platelet ($\times 10^3/mm^3$)	447 ± 63.37	443 ± 60.57
White Blood Cell ($\times 10^9/L$)	6.9 ± 5.12	5.86 ± 1.79
Lymphocytes (%)	59.67 ± 3.51	49.33 ± 6.51
Granulocytes (%)	40 ± 7.21	47.33 ± 7.02
Monocytes (%)	2.33 ± 0.58	3.33 ± 0.58

creatinine in the test rats are not significantly different ($p > 0.05$) from the control rats. Similarly, the mean value of ALT and AST in test rats was not significantly influenced ($p > 0.05$) by the aqueous extract compared to control rats.

Physiological responses of rabbits fed graded levels of *M. oleifera* leaf meal on haematological and biochemical parameters

The results of the haematological parameters of rabbits fed different diets are shown in Table 4. RBC, WBC, Blood Platelets, Haemoglobin, MCV, MCHC, and MCH level showed no significant difference ($P > 0.05$) in different treatments compared to the control group.

The results of the effect of the *M. oleifera* leaf meal incorporated into the granulated feed of rabbits on the biochemical parameters are presented in Table 5. In fact, alanine aminotransferase, aspartate amino transferase, cholesterol, rabbit creatinine and urea in the different treatments showed no significant difference ($p > 0.05$). However, there is a slight increase of urea of subjects treated compared to the control. Similarly, a non-

Table 3. Effect of *Moringa oleifera* extract on biochemical parameters in rats.

Parameter	Extract	Control
Urea (mg/L)	0.70±0.11	0.70±0.20
Creatinine (g/l)	6.18±0.27	5.65±0.43
AST (UI/L)	121.69±4.64	101.54±19.17
ALT (UI/L)	70.07±8.44	63.62±6.85

Table 4. Effect of *Moringa oleifera* leaf meal on haematological parameters in rabbits.

Parameter	Diets				LS
	T_0 (0% *M. oleifera*)	T_5 (5% *M. oleifera*)	T_{10} (10% *M. oleifera*)	T_{15} (15% *M. oleifera*)	
Red Blood Cell ($x10^6/mm^3$)	5.12 ± 0.90	5.14 ± 0.32	5.8 ± 0.65	5.51 ± 1.23	ns
Haemoglobin (g/dl)	9.85 ± 1.77	10.1 ± 0.83	11.63 ± 1.178	11.225 ± 2.81	ns
Packed Cell Volume (%)	33.65 ±4.67	34.7 ± 2.57	39.05 ± 3.20	37.4 ± 9.17	ns
Mean corpuscular volume (fl)	66.23 ± 2.96	67.8 ± 4.84	67.65 ± 3.80	67.7 ± 3.6	ns
Mean Corpuscular Haemoglobin (pg)	19.2 ± 0.41	19.6 ± 1.01	20.03 ± 0.70	20.2 ± 0.83	ns
Mean Corpuscular Haemoglobin Conc. (g/dl)	29.08 ± 1.45	29 ± 0.88	29.7 ± 0.80	29.83 ± 0.88	ns
Platelet ($x10^3/mm^3$)	238.5 ± 35.50	231.5 ± 22.40	227.5 ± 26.04	221.75 ± 48.22	ns
White Blood Cell ($x10^9/L$)	5.1 ± 1.99	6.48 ± 1.90	5.1 ± 0.82	5.15 ± 1.61	ns
Lymphocytes (%)	47 ± 12.68	30.5 ± 12.56	36.25 ± 12.69	30.25 ± 10.05	ns
Granulocytes (%)	49.75 ± 12.07	66 ± 12.19	60.5 ± 12,48	30.25 ± 10.05	ns
Monocytes (%)	3 ± 0.817	3.5 ± 1	3.25 ± 0.5	2.75 ± 0.5	ns

LS = Level of significance; ns = not significant.

Table 5. Effect of *Moringa oleifera* leaf meal on biochemical parameters in rabbits.

Parameter	Diets				LS
	T_0 (0% *M. oleifera*)	T_5 (5% *M. oleifera*)	T_{10} (10% *M. oleifera*)	T_{15} (15% *M. oleifera*)	
Glucose (mg/dl)	95.74 ± 0.26^a	98.64 ± 0.42^b	98.77 ± 0.62^b	98.05 ± 0.33^b	s
Urea (g/L)	0.29 ± 0.07	0.45 ± 0.08	0.53 ± 0.10	0.57 ± 0.34	ns
Creatinine (g/L)	14.11 ± 0.66	9.53 ± 2.36	10.36 ± 2.39	12.43 ± 3.56	ns
Cholesterol (g/L)	0.99 ± 0.55	0.75 ± 0.23	0.86 ± 0.48	0.87 ± 0.25	ns
Total Protein (g/L)	6.51 ± 1.37	7.76 ± 1.55	8.78 ± 0.46	6.73 ± 0.50	ns
Albumin (g/L)	4.07 ± 0.70	3.94 ± 0.26	4.15 ± 0.07	4.08 ± 0.06	ns
AST (U/l)	67.5 ± 26.81	59.5 ± 16.52	66.75 ± 22.75	61.5 ± 16.34	ns
ALT (U/l)	78 ± 26.14	59 ± 17.60	71.5 ± 27.68	58.75 ± 12.12	ns
ALP (U/l)	122.22 ± 2.46	115.46 ± 5.57	125.35 ± 4.06	118.89 ± 7.94	ns

AST= Aspartate Aminotransferase; ALT= Alanine Aminotransferase ALP= Alkaline phosphatase. LS = Level of significance; ns = not significant.

significant decrease in cholesterol levels is noted in the subjects treated relative to the control.

DISCUSSION

The acute oral toxicity of *M. oleifera* leaves meal was evaluated in this study to determine the tolerance limits of this plant just as used by traditional healers. Indeed, two weeks after the administration of the aqueous extract of *M. oleifera* leaf meal at the single dose of 2000 mg/kg body weight to the rats, no mortality within the batches was recorded. In addition, no adverse effects such as seizures, agitation, diarrhea, tremors, breathing difficulties

and hair loss were observed within 14 days post-treatment. A significant difference was observed in body weight of animals in both the control and test groups. This weight gain observed in the animals of the test batch may be due to a beneficial action of the plant extract inducing an increase in appetite of rats causing them to increase their daily ration. In addition, it has been reported that, in addition to their therapeutic value, medicinal plants can also positively affect the nutritional status of animals (OECD, 2001).

According to Dougnon et al. (2013), any plant whose toxicity does not exceed 1000 mg/kg is said to be nontoxic and in this present study, the extract was administered at a dose of 2000 mg/kg. The aqueous extract of *M. oleifera* leaf meal administered at a single dose of 2000 mg/kg body weight had no significant effect on the hematological parameters of the rats. Therefore, the aqueous extract from the plant could have an effect against anemia, enhance the immune system and facilitate blood clotting for increased blood platelet levels. Moreover, no deformation of the appearance of Red Blood Cells and White Blood Cells has been observed and this further reveals the non-toxic nature of plant extracts (Oduola et al., 2007). Aminotransferases (AST and ALT) are biomarkers of liver malfunction and can be used to evaluate liver cytolysis with ALT as being a more sensitive biomarker of hepatotoxicity than AST (Pramyothin et al., 2006). In our study, transaminase levels (AST and ALT) that reflect liver function status did not significantly change the test batch compared to the control group. This is an indication that the extract did not affect normal liver function. In addition, a significant increase or decrease in transaminase activity, especially ALT, is often associated with evidence of hepatocellular damage (Wannang et al., 2007). *M. oleifera* leaf meal are non-toxic in rats at a dose of 1000 mg/kg (Jonathan et al., 2014). In addition, no significant difference, of plasma glucose, cholesterol and creatinine levels were noted in test rats compared to control rats. These results converge with those of Akouango et al. (2014) who evaluated the acute toxicity of *M. oleifera* in mice orally at doses ranging from 100 to 5000 mg/kg orally (PO) and from 10 to 2000 mg/kg intraperitoneal (PI). The results obtained for the acute oral toxicity test on the aqueous extract of *M. oleifera* leaf meal made it possible to affirm that the oral use of the leaves of this plant seems relatively safe.

The analysis of blood parameters is a means of establishing the state of health of an animal and thus determining the effect of the ingestion of food components on the blood composition (Church et al., 1984). Blood testing can therefore be considered an appropriate measure of long-term nutritional status.

Iheukwuemere et al. (2006) have shown that changes in hematological and biochemical parameters in animals indicate their physiological state. Thus, the hematological components are useful in the monitoring of food toxicity especially with feeding components, which may affect the formation of blood. PCV is a parameter for measuring relative blood mass. PCV levels obtained in this study are not significantly ($p>0.05$) influenced by different dietary treatments. Their values were in the 33 to 50% reference range considered normal Packed Cell Volume of a healthy rabbit reported by Burns and De Lannoy (1966). The normal value of hematocrit shows the proper nutritional status of rabbits (Church et al., 1984). The result of our study is in agreement with the conclusion of Jiwuba et al. (2016) who observed no significant difference ($p>0.05$) in the PCV of rabbits fed *M. oleifera* leaf meal. There is no significant difference in the number of red blood cells (specialized blood cells in oxygen transport) in this study which is in the reference range (5.46×10^{12} to 7.94×10^{12} L^{-1}) according to Mitruka and Rawnsley (1977). This result corroborates that of Jiwuba et al. (2016), who also found no significant influence of feeding in the red blood cells of rabbits fed *M. oleifera* leaf meal. The hemoglobin levels obtained showed no significant difference ($p>0.05$) with respect to the control. However, the hemoglobin levels of the treatments are slightly higher than that of the control. Overall, it should be noted that these levels are within the reference range (9.40 to 17.90 g/dL) according to Campbell (2015). MCV, MCHC, and MCH values obtained in this study are not affected by the different dietary treatments and are included in the normal values of healthy rabbits. These parameters being important morphological characters of anemia (Campbell, 2015), show that rabbits are not anaemic. The White Blood Cell was not significantly ($p>0.05$) influenced by dietary treatments. The values obtained in this study are within the reference values recommended by Campbell (2015). These values show that rabbits fed with the gradual concentrations of *M. oleifera* leaf meal in the diet are in good health. Indeed, leukopenia is an indicator of allergies, anaphylactic shock, and certain parasitism, while the high number of white blood cells indicates the existence of a recent infection (Ahamefule et al., 2008). Granulocytes, lymphocytes and monocytes do not have a significant difference ($p> 0.05$). This indicates a probably normal physiological state in these rabbits subjected to these different treatments. The presence of monocytes in rabbit blood with the different treatments contradicts the results of the works of Bitto et al. (2006) that recorded the complete absence of monocytes in rabbits.

The biochemical results reveal no significant difference ($p> 0.05$) with respect to the control. The values of urea, creatinine, alanine aminotransferase, aspartate aminotransferase and cholesterol are included in the reference values. The absence of a significant difference in transaminases shows the protective hepatotoxic effect of *M. oleifera* leaves on the health of rabbits. The absence of significant differences in creatinine and urea also shows the nephroprotective property of *M. oleifera* leaves on the health of rabbits.

These results show that *M. oleifera* leaves can be incorporated into the granulated feed of rabbits up to 15% without any deleterious effect on the health of the rabbits if the good hygienic practices are maintained.

Conclusion

This study indicated that oral administration of aqueous extract of *M. oleifera* leaf at 2000 mg/kg body weight showed no changes in clinical signs and blood parameters. Therefore, *M. oleifera* have no toxicity at 2000 mg/kg body weight. From this study, it was concluded that *M. oleifera* leaf meal did not affect the biochemical and haematological parameters of rabbits. *M. oleifera* leaf meal may be incorporated into the rabbit's feed formulation at 15% rate. *M. oleifera* leaf meal can be used for increase in growth and health performance of rabbits and also subsidiary income of breeders.

Ethical committee approval

The study was carried out in strict compliance with the recommendations of the guide of the Research Ethics Committee of the National University of Agriculture (UNA), Porto Novo, Republic of Benin and in line with detailed protocols of Animal Care and Use in Research, Education and Testing: N° 062- 2016/ P-Ethic Committee/SA

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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