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Possible Immunological Alterations in the Plasma Levels of TNF-α and IL-10 in the Traditional Application of *Vernonia Amygdalina* (Ewuro) Leaves in the Treatment of Diabetes Mellitus

Mathew F. Olaniyan[1]*, Alade A. Ogunlade[2], David Atere[3]
[1]Department of Medical Laboratory Science, Edo University, Iyamho, Nigeria.
[2]Department of Nursing Science, Edo University, Iyamho, Nigeria.
[3]Department of Medical Laboratory Science, Achievers University, Owo, Nigeria.

***Correspondence:** olaniyanmat@yahoo.com; olaniyanmat@gmail.com

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Abstract
Diabetes mellitus can induce release of free radicals and oxidative stress, which can trigger production of cytokines. *Vernonia amygdalina* has antidiabetic activity due to its phytochemical constituents. This work was designed to determine possible immunological alterations in the plasma levels of TNF-α and Interleukin-10 (IL-10) in the traditional application of *V. amygdalina* (Ewuro) leaves in the treatment of diabetes mellitus. The study populations include all 33 diabetes mellitus patients (51-67 years; male, 21; female, 12) who had not commenced any form of medication or treatment and received herbal treatment in 10 herbal homes of Saki West, a local government area in Nigeria. Twenty-seven age-matched volunteers who were treated on insulin medication in the hospital almost within the same period were also investigated. Patients who were positive to acid-fast bacilli sputum test, *Plasmodium spp.*, identification, HBsAg, anti-HCV, and HIV-1 p24 assays were not included. Plasma TNF-α, IL-10, HBsAg, anti-HCV, and HIV-1 p24 were determined in the patients by ELISA while identification of acid-fast bacilli and *Plasmodium spp.* were carried out by Ziehl-Neelsen and Giemsa thick blood-film staining, respectively. There was a significant decrease in the plasma levels of TNF-α, IL-10, and glucose in diabetes mellitus patients after the administration of raw liquid extract of *V. amygdalina* and insulin compared to their basal samples before the commencement of the treatment ($p < 0.05$). The work revealed a significant increase in plasma TNF-α, IL-10, and glucose in diabetes patients, which returned to normal plasma values after treatment using raw liquid extract of *V. amygdalina* leaves and insulin.

Keywords: Immunochemical alterations; TNF-α; IL-10; *Vernonia amygdalina* leaves; Diabetes mellitus.

1. INTRODUCTION

Diabetes mellitus is a metabolic disease characterized by high blood-glucose levels over a prolonged period [1]. This can be due to either low insulin production by pancreas, or the cells of the body are not responding properly to the insulin produced [1-3]. It can occur in pregnancy when pregnant women with no history of diabetes mellitus present with high blood-glucose levels, which my persist or return to normal after delivery [1-3]. The symptoms include polyuria, polydypsia, and polyphagia [1-3]. Possible complications if untreated include diabetic ketoacidosis, hyperosmolar hyperglycemic state or death, cardiovascular problems, diabetic gangrene, muscle waste, stroke, kidney disease, foot ulcers, and vision impairment [1-3].

Inflammatory responses involves immune-modulating and cell, paracrine, and endocrine signaling agents such as TNF-α and Interleukin-10 (IL-10) generally referred to as cytokine [4, 5]. TNF-α is a proinflammatory cytokine and one of the cytokines of acute phase reaction. TNF-α is an endogenous pyrogen that induces fever, insulin resistance, apoptotic cell death, cachexia, and inflammation and inhibits tumorigenesis and viral replication [6, 7]. IL-10, which is an anti-inflammatory cytokine, inhibits production of inflammatory cytokines such as TNF-α [4, 5].

Oxidative stress plays a major role in the pathogenesis and development of complications of diabetes mellitus because hyperglycemia can induce free radicals and impair the endogenous antioxidant defense system in patients with diabetes mellitus [8]. Many inflammatory cytokines are induced by oxidative stress, as cytokines can also trigger the release of other cytokines, which can lead to increased oxidative stress [4, 5].

Raw liquid content of *Vernonia amygdalina* (Ewuro) leaves is one of the common leaf extracts used to treat diabetes in Nigeria [9-15]. The possible antidiabetic property of *V. amygdalina* could be associated with the fact that the aqueous extract has been known to enhanced glucose utilization and uptake [10]. The leaves contain vitamins A, C, E, B1, B2; saponins; flavonoids; alkaloids; terpenes; steroids; coumarins; phenolic acids; lignans; xanthones; anthraquinones; edotides; and sesquiterpenes [16-19].

Therefore, this work was designed to determine possible immunological alterations in the plasma levels of TNF-α and IL-10 after the traditional application of *V. amygdalina* (Ewuro) leaves in the treatment of diabetes mellitus.

2. METHOD(S)

2.1. Study Area
The study area includes 10 herbal homes in Saki West, a local government area located in the northern part of Oyo State in Nigeria. It shares borders with Burkina Faso and Kwara State, Nigeria. It hosts institutions like the resettlement center of the second mechanized division of the Nigerian Army, the Oke-Ogun Polytechnic, and a technical college.

2.2. Population of the Study
The study populations include all 33 diabetes mellitus patients (51-67 years; male, 21; female, 12) who had not commenced any form of medication or treatment and received herbal treatment in 10 herbal homes of Saki West, a local government area in Nigeria, between February and July 2018. Twenty-seven age-matched volunteers who were placed on insulin medication at Baptist Medical Centre, Saki Nigeria, almost within the same period were also investigated. Patients who were positive to acid-fast bacilli sputum test, *Plasmodium spp.,* identification, HBsAg, anti-HCV, and HIV-1 p24 assays were not included.

2.3. Biological Sample
Early morning sputum was obtained from each patient for Ziehl-Neelsen staining to demonstrate acid-fast bacilli. Fasting venous blood sample was also obtained from each of the patients for the determination of fasting blood glucose, plasma TNF-α, IL-10, HBsAg, anti-HCV, and HIV-1 p24 before and after treatment.

2.4. Preparation and Administration of Raw Liquid Extract of Vernonia Amygdalina (Ewuro) Leaves
Fresh leaves of *V. amygdalina* (Ewuro), plucked on a daily basis, were confirmed by the Department of Agriculture Technology, Oke-Ogun Polytechnic, Saki, Oyo State, Nigeria. The leaves were washed and squeezed to extract the raw liquid content; 70 ml of the liquid extract was administered orally to each of the diabetes patients on a daily basis for about 11 days when the high blood glucose reduced to normal plasma levels.

2.5. Treatment Administered in the Hospital
Those who received treatment in the hospital were given insulin injection at an average dose of 0.6 units/kgBW/day.

2.6. TNF-α ELISA
Plasma TNF-α was determined in the subjects using Abcam's kit, USA. Abcam's samples, standards, and control specimens were pipetted into microtiter wells coated with monoclonal antibody-specific TNF-α. This preparation was incubated and washed and was then followed by the addition of the enzyme streptavidin-HRP that binds the biotinylated antibody, incubated and washed again. A TMB substrate solution was added as a substrate to the bound enzyme for color generation. The intensity of this colored product is directly proportional to the concentration of TNF-α present in the samples.

2.7. IL-10 ELISA
Samples, standards, and control specimens were pipetted into microtiter wells coated with monoclonal antibody-specific IL-10. This preparation was incubated and washed and was then followed by the addition of the enzyme streptavidin-HRP that binds the biotinylated antibody, incubated and washed again. A TMB substrate solution was added as a substrate to the bound enzyme for color generation. The intensity of this colored product is directly proportional to the concentration of IL-10 present in the samples.

2.8. HIV-1 p24 Antigen ELISA Using Zeptrometrix Retrotek Kit, Corporate Headquarters, 878 Main Street Buffalo, New York 14202
Principle: Microtiter wells were precoated with a monoclonal antibody specific to the p24 gag gene product of HIV-1. Viral antigen in the sample is specifically bound onto the immobilized antibody during specimen incubation. The bound antigen then reacted with a high-titered human anti-HIV-1 antibody conjugated with biotin. Following a subsequent incubation with streptavidin-peroxidase, color develops as the bound enzyme reacts with the substrate. The intensity of the color produced is proportional to the amount of HIV-1 p24 antigen present in the specimen.

2.9. Anti-HCV ELISA Assay
This was determined by using Anti-Hepatitis C Virus Core Antigen antibody (ab50288) Abcam USA kit.

2.10. HBsAg ELISA Test
This was assayed using diagnostic automation, Cortez Diagnostics, INC, USA kit, by ELISA method.
Principle: The HBsAg ELISA Test used an antibody sandwich ELISA technique where monoclonal antibodies specific to HBsAg were precoated on microtiter polystyrene strips. The plasma sample, control, and standard were then added. After incubation

and washing to eliminate unwanted serum proteins and unbound HRP-conjugates, chromogen solutions that contain tetramethyl-benzidine (TMB) and urea peroxide were added to the wells. The colorless chromogens were hydrolyzed by the bound HRP-conjugate to a blue-colored product. Sulfuric acid was added to stop the reaction and the blue color then turned yellow. This color intensity is directly proportional to the concentration of the antigen in the samples. If the blue color remains colorless, it indicates HBsAg negative.

2.11. Determination of Acid-Fast Bacilli in Sputum

Ziehl-Neelsen (Zn) staining technique was used to identify acid-fast bacilli in the sputum sample (*Mycobacterium species)*, as described by Cheesbrough [20].

2.12. Identification of Plasmodium Species Giemsa-Staining Thick Blood-Film Technique

Plasmodium species were identified by using Giemsa thick blood-film staining technique as described by Cheesbrough [20].

2.13. Fasting Blood Glucose

This was analyzed in each of the patients by glucose oxidase method using the reagent kit of Randox, Crumlin, County Antrim.

2.14. Ethical Consideration

Ethical approval for this work was obtained from ethical and research committee of Baptist Medical Centre, Saki, Nigeria, before the commencement of work. Informed consent was also obtained from each of the patients.

2.15. Data Analysis

Data were analyzed for mean, standard deviation, t test, and probability values at 0.05 level of significance using the statistical package for social sciences (IBM SPSS, version 18).

3. RESULTS

There was a significant decrease in the plasma levels of TNF-α, IL-10, and glucose in diabetes mellitus patients after the administration of raw liquid extract of *V. amygdalina* and insulin compared to their basal samples before the commencement of the treatment ($p < 0.05$, Tables 1 and 2, Figures 1 and 2).

Table 1: Mean and standard deviation of plasma TNF-α, IL-10, and glucose in diabetes mellitus patients before and after the administration of raw liquid extract of *Vernonia amygdalina* and insulin.

Cytokines	DM patients on bitter leaf extract ($n = 33$)		DM patients on insulin injection ($n = 27$)	
	Before treatment	After treatment	Before treatment	After Treatment
TNF-α (pg/ml)	4.1 ± 0.2	2.7 ± 0.1	4.5 ± 0.1	2. 4 ± 0.1
IL-10 (pg/ml)	5.9 ± 0.1	4.4 ± 0.2	6.8 ± 0.2	3.9 ± 0.1
Glucose (mg/dl)	201 ± 2.0	111 ± 3.0	297 ± 5.0	115 ± 2.0

Table 2: Comparative analysis of plasma TNF-α, IL-10, and glucose in diabetes mellitus patients before and after the administration of raw liquid extract of *Vernonia amygdalina* and insulin.

Cytokines		DM patients on bitter leaf extract ($n = 33$)	DM patients on insulin injection ($n = 27$)
		After vs. before treatment	After vs. before treatment
TNF-α (pg/ml)	*t* value	−6.26099.	−14.84924
	p value	0.012*	0.0023*
IL-10 (pg/ml)	*t* value	−6.7082	−12.96919
	p value	0.011*	0.003*
Glucose (mg/dl)	*t* value	−24.96151	−33.79655
	p value	0.0008*	0.0004*

NB: *Significant

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Figure 1: Comparative description of plasma TNF-α, and IL-10 in diabetes mellitus patients before and after the administration of raw liquid extract of *Vernonia amygdalina* and insulin.

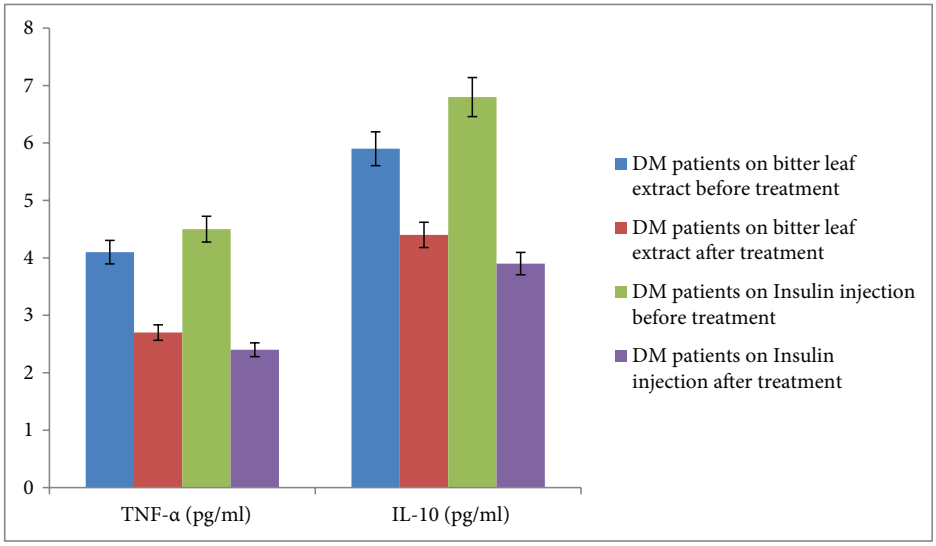
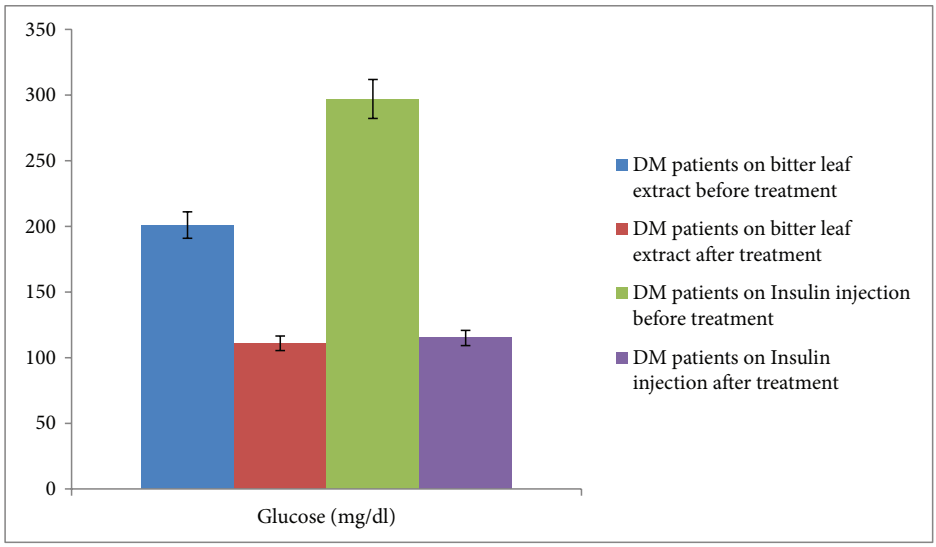


Figure 2: Comparative description of plasma glucose in diabetes mellitus patients before and after the administration of raw liquid extract of *Vernonia amygdalina* and insulin.



4. DISCUSSION

The result obtained in this work showed a significant decrease in the plasma levels of TNF-α, IL-10, and glucose in diabetes mellitus patients after the administration of raw liquid extract of *V. amygdalina* and insulin compared to their basal samples before the commencement of the treatment.

These findings could be attributed to the facts discussed in the following paragraphs.

The antidiabetic bioactivity of *V. amygdalina* has been reported and associated with to the ability of the aqueous extract to enhance glucose utilization and uptake, which is responsible for the reduction in the high fasting blood-glucose levels after the patients were treated with the raw liquid extract of *V. amygdalina* leaves [10]. This could also be associated with the phytochemical and the phytonutrient constituents, which include vitamins A, C, E, B1, B2; saponins; flavonoids; alkaloids; terpenes; steroids; coumarins; phenolic acids; lignans; xanthones; anthraquinones; edotides; and sesquiterpenes [16-19].

High plasma concentration of TNF-α and IL-10 in diabetic patients before treatment could be related to the fact that oxidative stress is involved in the pathogenesis and development of complications of diabetes mellitus, as hyperglycemia can generate free radicals and impair the endogenous antioxidant defense system in patients with diabetes mellitus [8].

In addition, raised plasma TNF-α could be triggered by oxidative stress caused by diabetes mellitus [8], resulting in an inflammatory response, as TNF-α is a proinflammatory cytokine. IL-10 is an anti-inflammatory cytokine that prevents the secretion of proinflammatory cytokines like TNF-α. The raised plasma level of IL-10 in this work could be associated with the normal immunological responses of IL-10. Furthermore, oxidative stress can induce many inflammatory cytokines. Cytokines can trigger the release of other cytokines, which can also increase oxidative stress [4, 5].

Decrease in plasma levels of TNF-α, IL-10, and glucose in diabetic patients after the administration of raw liquid extract of *V. amygdalina* and insulin could be linked with the fact that hyperglycemia can stimulate production of free radicals and cause oxidative stress that can trigger the release of cytokines [4, 5, 8], which in turn leads to the initial increase of the two cytokines, but their levels decrease, as a result of decrease in initially raised blood-glucose levels following the administration of raw liquid extract of *V. amygdalina* and insulin. This can also be associated with the possible anti-inflammatory activity of raw liquid extract of *V. amygdalina* [16-19].

5. CONCLUSION

The worked revealed a significant increase in plasma TNF-α, IL-10, and glucose in diabetes patients, which returned to normal plasma values after treatment using raw liquid extract of *V. amygdalina* leaves and insulin.

Author Contributions

This work was carried out with the collaboration of all the authors. Mathew F. Olaniyan was responsible for the design, sample collection, data analysis, literature search, and preparation of the research report. In addition, Alade A. Ogunlade carried out the sample collection, literature search, and preparation of the research report, and David Atere performed the sample collection, data analysis, and literature search.

Conflict of Interest

None.

Funding

None.

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