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Regulatory Activity of Ethanol Leaves Extract of Moringa Oleifera on Benzene Induced Leukemia in Wister Rat Using TNF-α Analysis

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Abstract Medicinal plants are important elements of indigenous medicinal system that have persisted in developing countries. Compounds that are capable of interacting with the immune system to up regulate or down regulate specific aspects of the host can be classified as immunomodulators. This study aimed at determining the regulatory activity of ethanol extract of *Moringa oleifera* in leukemic wistar rats using Tumuor necrosis factor assay. Thirty adult wistar rats were divided into three groups each containing ten wistar rats. Intravenous injection of 0.2 mg/ml of benzene chromosolv was administered 48 hourly for four weeks. 0.2 ml of 100 mg/ml of ethanol extract of *Moringa oleifera* was administered orally pre, during and post leukemia induction. Group A (Normal rats), Group B (benzene administration only) and Group C (benzene + ethanol *Moringa oleifera* extract). After a total period of eight weeks, the plasma samples of each of the groups was collected and analyzed using TNF-α kit by ELISA technique. The result showed that there was no significant difference in TNF-α levels between all the groups. However there was a mean ± standard deviation difference between the groups; the group of rats induced with leukemia without treatment (leukemia control) with 229.38 ± 58.17 ng/L, those treated with EMO after induction of leukemia had a mean and standard deviation of 219.38 ± 18.52 ng/L while the normal control group 209.04 ± 17.51 ng/L. This study concluded that the anti-cancer properties of Ethanol extract of *Moringa oleifera* results in the down regulation of TNF-α as treatment of cancer occurs. Also TNF-α level may be indicative of the clinical efficacy of therapy.

Keywords: *moringa oleifera, wistar rats, TNF-α, ELISA, ethanol extract, benzene chromosolv*

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1. Introduction

The immune system defends the body from attack by invaders recognized as foreign. It is an extraordinary complex system that relies on an elaborate and dynamic communications network that exists among the many different kinds of immune system cells that patrol the body. At the heart of the system is the ability to recognize and respond to substances called antigens whether they are infectious agents or part of the body [1]. The immune system is characterized by three universal features namely specificity, diversity, and memory [2]. Most immune system cells are white blood cells, of which there are many types [1].

The immune system does not only combat foreign antigenic substances but is also vigilant in identifying and eliminating nascent tumor cells. And a special type of immune cells called the T-cells is pivotal sentinel in the immune system's response against cancer. This is known as immunosurveillance [3]. Cancer immunology is the study of interactions between the immune system and cancer cells (also called tumors or malignancies). Immunoediting is a process by which a person is protected from either cancer growth or the development of tumor immunogenicity by their immune system [4].

Tumor-specific T-cells can be activated to inhibit tumor growth [5]. T-cells during its immunologic response to tumors secrete tumor necrosis factor (TNF). Also cells expressing CD4 secrete TNF (3).

In contrast to chemotherapeutic drugs, TNF specifically attacks malignant cells. Extensive preclinical studies documented a direct cytostatic and cytotoxic effect of TNF against subcutaneous human xenograft and lymph node metastasis in immunodeficient mice, as well as a

variety of immunomodulatory effects on various immune effector cells, including macrophages and T-cells. TNF can be administered safely to patients with advanced malignancies in a dose range associated with anticancer effect without concomitant serious toxicities such as shock and cachexia. It is an antineoplastic agent in most cases. Cancer can also be induced by extraneous substances [6].

Hundreds of chemicals are capable of inducing cancer in humans or in animals after prolonged or excessive exposure. There are many well-known examples of chemicals that can cause cancer in humans. Leukemia can result from chemically induced changes in the bone marrow from exposure to benzene and cyclophosphamide, among other toxicants [7].

Benzene-induced leukemia has been proposed to occur due to the following turn out of events within humans or animals, namely; Activation of benzene in the liver to phenolic metabolites; Transport of the metabolites to the bone marrow and conversion to semi Quinone radicals and quinones via peroxidase enzymes; Generation of active oxygen species via redox cycling; Damage to tubulins, histone proteins, topoisomerase II, other DNA associated proteins, and DNA itself; and Consequent damage including DNA strand breakage, mitotic recombination, chromosome translocations, and aneuploidy. This process takes place in the stem or early progenitor cells hence a leukemic clone with selective advantage to grow may arise, as a result of proto-oncogene activation, gene fusion, and suppressor gene inactivation [8].

Immunomodulation refers to the changes in the body's immune system, caused by agents that activate or suppress its function [9]. The concept of immunomodulation relates to nonspecific activation of function and efficiency of macrophages, granulocytes, complement, natural killer cells and lymphocytes and also to the production of various effector molecules generated by activated cells. It is expected that this non-specific effect give protection against different pathogens including bacteria, viruses, fungi etc. and constitute an alternative to conventional chemotherapy [10].

Medicinal plants are being used in traditional system of medicine from hundreds of years in many countries of the world [11]. In recent times, the interest in medicinal plants has increased in a great deal due to its therapeutic properties, which is very useful in healing various diseases and the advantage of the plant being 100% natural [12].

Plants are the essential and integral part in complementary and alternative medicine and due to this they develop the ability for the formation of secondary metabolites like proteins, flavonoids, alkaniods, steroids, and phenolic substances which are in turn used to restore health and heal many diseases. Immunomodulatory activities have been reported in numerous plants [10]. One of such plants is *Moringa oleifera* (MO).

Moringa (Moringa spp.) is a softwood perennial tree that belongs to the monomeric family Moringaceae. *Moringa oleifera* commonly known as drumstick tree, Ben oil tree, Horseradish tree [13] is most commonly cultivated in south India, Ethiopia, Philippines, Sudan and other tropical countries [14]. The leaves are believed to have a stabilizing effect on blood pressure and glucose levels. They are also used to treat anxiety, diarrhea and inflammation of the colon, skin infection, scurvy, intestinal parasites, and many other conditions. The root is a laxative, expectorant, diuretic and good for inflammations (anti-inflammatory effect), throat bronchitis, piles, cures stomatitis, urinary discharge and chronic asthma [15]. *Moringa* leaves contain all of the essential amino acids which are the building blocks of protein in a good proportion, so that they are very useful to our bodies. It contains important amino acid required in infants, that is, arginine and histamine [16].

Water, methanol, and ethanol extracts of freeze-dried leaves of *Moringa oleifera* were examined for radical scavenging capacities and antioxidant activities. And it was found that all leaf extracts were capable of scavenging peroxyl and superoxyl radicals. Ethanol extracts and methanol have the highest antioxidant activities. The major bioactive compounds of phenolics are found to be flavonoid groups such as quercetin and kaempferol [17].

In particular, the plant is rich in compounds containing anticancer activity which includes 4-(4'-0-acetyl-L-rhamnopyranosyloxy) benzyl isothiocyanate (2) and 4-(-L-rhamnopyranosylox) benzyl glucosinolate (4). It has been experimented to act as a protective agent inflammation associated with development of arthritis in rats [18].

Previous experimental works on the treatment of cancer has shown that the use of chemotherapeutics have some degree of detrimental effects on normal body cells hence resulting in pain to patients. They also result in toxic effects that supersede their therapeutic purpose. This has stimulated a rationale on making findings on a possible alternative therapy that won't be of detrimental effect to patients. Thus, the use of medicinal plants extracts and metabolites which are of naturally occuring products and non-toxic. They are used to stimulate the immune system and also exert a direct anti-cancer effect activity on cancer cells in the treatment of cancer. *Moringa oleifera* is an example of such plants. Studies have shown that the extracts of *Moringa oleifera* possess anticancer activity. This study therefore aims at investigating the regulatory activity of ethanol *Moringa oleifera* leaves extract in leukemia using Tumor necrosis factor analysis.

2. Materials and Methods

2.1. Materials

Wister rats, Extract from *Moringa oleifera*, Wooden cages, oral cannular, Hand gloves, K3 EDTA bottles, Needles and syringes, blades, Cotton wool and methylated spirit, Commercial feed and water, Plain bottles, Permanent marker, Measuring cylinder, Whatman filter paper, Distilled water.

2.1.1. Reagents

Ethanol, Benzene-Chromosolv (Leukemic agent) was obtained from sigma, st. Louis, Rat Tumor necrosis factor α (TNF -α) ELISA Kit (Standard (1280ng/L, Standard diluent, Micro elisa strip plate, Str-HRP-Conjugate Reagent, 30×wash solution, Biotin-TNF-α Antibody) was obtained from Hangzhou EastBiopharm CO.,LTD, China, K3 EDTA was a product of British Drug house (Pooke, England).

2.1.2. Equipments

The major equipments used in this study are as follows: Weighing balance and Bench centrifuge were products of Lamfied and Gallenkamp, England respectively. Microtitre Plate washer, Automatic Multi-channel Micropipette, Microtitre Plate reader (Hangzhou EastBiopharm CO., LTD, China), ELISA Machine (China).

2.2. Methods

2.2.1. Experimental Rats

Thirty adult wistar rats with an average weight of 150 g were obtained from the animal house of the college of Health Sciences, Ladoke Akintola University of Technology, Osogbo. The rats were randomly arranged in separate wooden cages each containing 10 wistar in a group. They were allowed to acclimatize for a period of seven days before the commencement of the experiment. The animals were maintained at room temperature 28°C (±2) with 12 hours light/dark cycle and also allowed unrestricted access to water and rat feed.

2.2.2. Production of Ethanol Extract of *Moringa oleifera* Leaves

The fresh leaves *Moringa oleifera* were collected from Osogbo local government area of Osun State. It was identified at the department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria. Thereafter authenticated by the Department of pharmacognosis, Obafemi Awolowo University, Ile-Ife, Osun State. The extraction process began by the drying of the fresh leaves at room temperature over a period of three weeks. The dried leaves were then grinded into fine powder using an electric blender. The fine powder was then weighed and then soaked with measured quantity of ethanol (for every 10 ml of ethanol, 1g of *Moringa oleifera* powder). The mixture was stirred with the aid of a stirrer and left for 48 hours. Thereafter it was filtered using filter papers in order to collect the filtrate from the mixture. The volume of the filtrate was measured and evaporated to dryness at temperature 45°C with the use of petri dish placed in oven for the collection of the plant extract. The eventual residue was carefully scrapped, weighed and refrigerated until further analysis was carried out.

2.2.3. LD_{50} of the Plant Extract

The lethal dose of 1585 mg/kg was adopted according to Awodele *et al*., 2012.

2.2.4. Administration of Plant Extract

The ethanol extract of *Moringa oleifera* was administered by gavage once daily. With the aid of oral cannular of 0.2ml of 100mg/ml was administered to the rats over a period of 4 weeks.

2.2.5. Administration of the Benzene Chromosolv

Benzene chromosolv, a product of Sigma-Aldrich with Cat No 270709 and ≥ 99.9% was diluted in water for injection at a concentration of 1ml of the benzene chromosolv to 9ml of water for injection. Exactly 0.2ml was administered through the vein at the tail 48 hourly for 4 weeks.

2.2.6. Experimental Protocol

GROUP A: diet only (Normal control)

GROUP B: Administration of benzene for 4 weeks and sacrificed thereafter.

GROUP C: Administration of benzene + Ethanol extract of *Moringa oleifera*.

2.2.7. Plasma Sample Collection

After eight weeks of the experiment, the animals were sacrificed by cervical dislocation and blood samples were collected via heart puncture into EDTA bottles and labeled according to the groups. Plasma for each group was obtained by centrifuging blood samples at 2000 rpm for 5 minutes. The plasma samples were then refrigerated until the day of analysis.

2.2.8. TNF Analysis Using Rat TNF-α ELISA Kit

Principle: The kit uses a double antibody sandwich enzyme linked immunosorbent assay (ELISA) to assay the level of Rat Tumor necrosis factor α (TNF-α) in samples. Addition of Tumor necrosis factor α (TNF-α) to monoclonal antibody enzyme well which was pre-coated with Rat Tumor necrosis factor α (TNF-α) monoclonal antibody was done, incubated, then the addition of Tumor necrosis factor α (TNF-α) antibodies labeled with biotin, and combined with streptavidin-HRP to form immune complex, further incubation was carried out and washing again to remove the uncombined enzyme. Upon the addition of Chromogen solution A, B, the color of the liquid changed to blue, and at the effect of an acid, the color finally became yellow. The chroma of colour and concentration of the Rat subsatance Tumor necrosis factor α (TNF-α) of samples were positively correlated.

2.2.9. Assay Procedure

1. A total of six standard dilutions were made using the protocol table below
2. Standard well: It was run in duplicates making a total of twelve wells which contains 50µl of standard solution and 50µl of Streptavidin-HRP.
3. Blank well: Blank well was made by adding nothing to the well.
4. Sample well: Samples well were made by adding 40µl of the test sample followed by then addition of 10µl of Biotin (TNF-α antibody) and 50µl of streptavidin-HRP.
5. The wells were shaking gently after being sealed by a membrane seal and incubated for 60 minutes at 37°C.
6. Confection: A wash solution was prepared by diluting 30 times the 30×washing concentrate with distilled water.
7. Washing: The membrane was removed carefully, the liquid drained and the remaining water was shaken away. The wells were washed five times using the wash solution prepared to remove unbound antibody.
8. After washing, 50µl of chromogen solution A and 50µl of chromogen B solution was added to all the wells (the colour of the liquid changes to blue), gently mixed and incubated for 10 minutes at 37°C away from light.
9. Stop: 50µl of stop solution was added into each well to stop the reaction (the blue changes into yellow immediately).
10. Final measurement: The optical density (OD) was measured under 450 nm wavelength and was carried out within 15 minutes after adding the stop solution.

Table 1. Dilution Protocol

S/N	Concentration	Standard no	Dilutions
1	640 ng/L	5	120 µl Original Standard + 120 µl Standard Diluents
2	320 ng/L	4	120 µl Standard No. 5 + 120 µl Standard Diluents
3	160 ng/L	3	120 µl Standard No. 4 + 120 µl Standard Diluents
4	80 ng/L	2	120 µl Standard No. 3 + 120 µl Standard Diluents
5	40 ng/L	1	120 µl Standard No. 2 + 120 µl Standard Diluents
6	0 ng/L	0	120 µl Standard Diluents only

2.2.10. Preparation of ethanol extract of *Moringa oleifera* leaves

Weight of the empty nylon = 10.1 g
Weight of nylon with *Moringa oleifera* leaves = 440.5 g
Weight of *Moringa oleifera* leaves = 430.4 g
Weight of empty bottle = 359.2 g
Weight of empty bottle with grinded *Moringa oleifera* leaves = 785.6 g
Weight of grinded *Moringa oleifera* leaves = 426.4 g

The grinded *Moringa oleifera* leaves were kept in a clean bottle and mixed with ethanol in ratio 1:10 for extraction (4000 ml of ethanol to 400 g of *Moringa oleifera* leave powder). The mixture was left for 48 hours, but was stirred at intervals for proper extraction. Using whatman filter paper, the mixture was filtered in order to obtain filtrate from the mixture. The volume of the filtrate was 2998 ml and the weight of the residue was 350.5 g. The filtrate was poured into Petri dishes to evaporate to dryness at temperature of 40°C in oven for the collection of the plant extract.

Weight of the empty universal bottle = 10.7 g
Weight of the gelly-like ethanol extract with bottle = 57.7 g
Weight of gelly-like ethanol extract = 47 g

2.2.11. Calculation of the Plant Extract Administered

One gram (1 g) of the extract was weighed and was dissolved into 10 mL of distilled water which gives the working solution; 1 in 10 of lethal dose is normal dose

$$Administered\ volume = \frac{normal\ dose}{1000} \times \frac{body\ weight}{stock\ solution}$$

$$= \frac{158.5 \times 150}{1000 \times 100}$$

$$= 0.2\ ml\ of\ 100\ mg/ml\ of\ ethanol\ extract\ of\ Moringa\ oleiferaleaves.$$

2.2.12. Statistical Analysis Method

Statistical analysis was performed using SPSS version 15 with the following statistical tools: mean, standard deviation, one- way variance analysis (ANOVA) and p-value. Differences at $p<0.05$ were considered to be statistically significant and the experimental values are as mean ± standard deviation by Thamhane's post hoc test.

3. Results

The yield of extraction process for ethanol extract of *Moringa oleifera* leaves and the effect of benzene and ethanol extract of *Moringa oleifera* on TNF-alpha levels in various experimental groups are shown in the figure.

3.1. Yield of Ethanol *Moringa oleifera* Leaves Extraction

The result of the extraction process of the plant extract is as follows; 100 g of the dry powder of *Moringa oleifera* was soaked in 1 litre of Ethanol and after proper sieving and drying, 11.75 g of gelly-like residue was obtained.

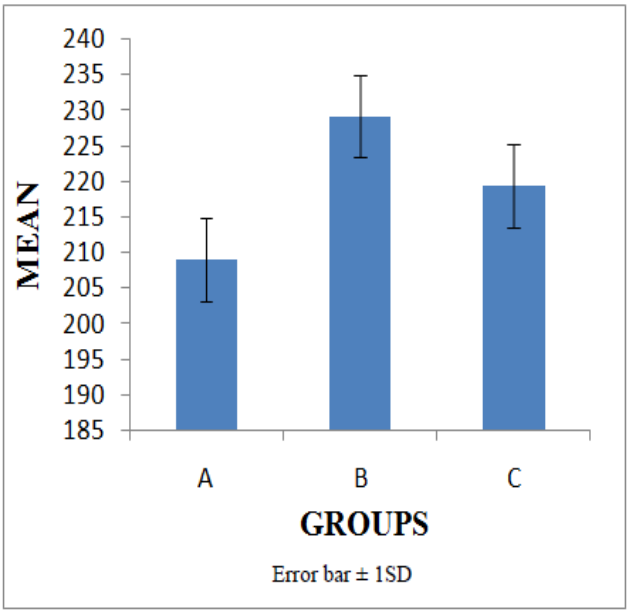

Figure 1. Bar chart showing comparison of TNF-alpha level in experimental groups (**Key**: S.D; Standard deviation; GROUP A; Normal control; GROUP B; leukemia control; GROUP C; Treatment group)

3.2. Effect of Benzene and Ethanol Extract of *Moringa oleifera* on TNF-alpha Levels

The results of the Tumor necrosis factor (TNF) – α level in the various experimental groups are shown in the figure below. In the group containing rats that were induced with leukemia without treatment, the TNF – α level was increased when compared with other groups. Figure 1 contains comparison of Group A given only rat chow and water which serves as the normal control, Group B treated with benzene for the induction of leukemia and Group C treated with benzene and then Ethanol *Moringa oleifera*. No statistical significant difference ($P > 0.05$) was observed between the groups, however there is a mean ± standard deviation difference in TNF – α production levels between them. Higher TNF – α level was seen in group B induced with leukemia without treatment greater than group induced with leukemia using benzene and then treated with Ethanol *Moringa oleifera* and normal control group. TNF – α level in the normal control group was less than that of the treatment group.

4. Discussion and Conclusion

The incidence of cancer in Nigeria appears to be low as compared to developed countries of the world which may not truly reflect the burden. The burden of cancer in Nigeria is unknown; mainly due to the lack of statistics or under reporting. This is not only peculiar to Nigeria but to most parts of Africa [19].

Understanding the mechanisms that control cell proliferation or apoptosis is essential to understanding and controlling diseases, such as acute myeloid leukemia. Monocytes and macrophages, which are derived from myeloid cells, have the ability to proliferate or die dependent on their environment and the controlling signals they encounter [20,21]. The concept of immunomodulation relates to nonspecific activation of function and efficiency of macrophages, granulocytes, complement, natural killer cells and lymphocytes and also to the production of various effector molecules generated by activated cells. It is expected that this nonspecific effects give protection against different pathogens including bacteria, viruses, fungi etc. and constitute an alternative to conventional chemotherapy [10]. Tumor necrosis factor α (TNF) is a proinflammatory cytokine produced mainly by activated macrophages or monocytes and plays an important role in diverse cellular events, such as the production of other cytokines, cell proliferation, differentiation and apoptosis [6].

This work studied the regulatory activity of Ethanol leaves extract of *Moringa oleifera* on benzene induced leukemic wistar rats using Tumor necrosis factor -alpha analysis (TNF-α). Benzene has been established to be a known carcinogenic substance that causes various forms of cancers, notably Acute Myeloid leukemia (AML), especially due to occupational hazards [2]. Leukemia was induced by the administration of 0.2 mg/ml of benzene chromosolv 48 hourly for four weeks. The induction of leukemia was confirmed by the estimation of a known tumor marker called Beta-2-Microglobulin (B2M). B2M level estimated in the various experimental groups, showed that there was marked increase in B2M level in the group of rats that were induced with leukemia without any treatment compared with the normal control group and those induced with leukemia but treated with Ethanolic extract of *Moringa oleifera* (Data not shown). This finding established the induction of Leukemia using benzene chromosolv. This result correlates with Campos *et al*., 1984 in which B2M level was found to be elevated above normal in acute myeloid leukemia patients. Also in previous works where higher values of B2M has been observed to be seen in malignant disorders particularly in multiple myeloma, monocytic leukemia and in patients with decreased glomerular filtration rate and also serial measurement of B2M is used to measure the tumor [22,23].

Results obtained from comparing the experimental groups, showed that there was no significant difference ($P > 0.05$) in the TNF-α level of the group of rats that were induced with leukemia, rats induced with leukemia and then treated with ethanol extract of *Moringa oleifera* (EMO) when compared with the group of rats that were not induced with leukemia at all (normal control). However mean ± standard deviation differences were observed between the groups. The group of rats induced with leukemia without treatment (Leukemia control) had 229.38 ± 58.17 ng/L, those treated with EMO after induction of leukemia had a mean ± standard deviation of 219.38 ± 18.52 ng/L while the normal control group had 209.04 ± 17.51 ng/L. This result shows that at the onset of leukemia, the immune system produces TNF-α in higher amount than in normal subjects in order to arrest the disease condition. However TNF-α level starts to decrease as treatment begins to take place but not lower than normal TNF – α level in normal subjects. This result is similar to those obtained when the changes and significance of tumor necrosis factor alpha (TNF α) in the serum and cerebrospinal fluid (CSF) of children with acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) were evaluated by Yu *et al* in 2003. *Moringa oleifera* has been shown to possess substantial anti-carcinogenic and anti-mutagenic activities due to their antioxidant and anti-inflammatory properties. According to Barak *et al* in 1999, decrease in TNF-alpha levels is indicative of the response of the subject to treatment while an increase in TNF- alpha levels accompanies active disease [20].

This study concludes by stating that the anti-cancer properties of Ethanol extract of *Moringa oleifera* results in the down regulation of TNF-α as treatment of cancer occurs. Also, TNF-α level may be indicative of the clinical efficacy of therapy.

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