CYTOTOXIC AND IMMUNOMODULATORY EFFECTS OF RICINUSCOMMUNIS LEAVES EXTRACT

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ABSTRACT

Background and Objectives: Traditionally, Ricinus communis has significant medical value for humans hence, they considered a promising anticancer agent, and regulate autoimmune diseases in addition to their traditional uses in different ailments. The goal of this in vitro study was to evaluate the cytotoxic effect of Ricinus communis leaves extract on isolated human PBMNs and Vero cell line, and to study the immunomodulatory effects of aqueous leaves extract on IL-17, IL-10, IL-17, and INF-γ secreted from PBMNs.

Materials and Methods: To evaluate the cytotoxic effects, isolated PBMNs and Vero cell line were grown in RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS) were treated with the ethanolic and aqueous plant extracts at different concentrations. Cells viability measured via MTT assay after an incubation period of 24 hours. The isolated PBMNs also treated with two concentrations of aqueous leaves extract of Ricinus communis to evaluate its immunomodulatory effects by measuring the levels of cytokines produced by PBMNs using ELISA technique.

Results: Unlike the aqueous extract, ethanolic extract showed a significant cytotoxic effect at all concentrations tested on Vero cell line and no effect on the viability of PBMNs. Aqueous extract showed a significant decrease in viability of PBMNs only at (1000 and 500) µg/ml. Aqueous leaves extract showed significant suppression of IL-17 secretion at a high concentration as compared to the negative and positive controls. Compared to the positive group, there was a significant decrease in IL-10 and IL-4 production at both concentrations, while INF-γ levels significantly reduced only at higher concentration. Conclusion: The aqueous leaves extract has cytotoxic effects on Vero cells and PBMN only at the higher concentrations, while ethanolic leaves extract has cytotoxic effects on Vero cells at all concentration used. Ricinus communis aqueous leaves extract showed a significant decrease in IL-17, IL-10, IL-4 and IFN-gamma. This suggests a potential use of this extract in autoimmune diseases such as asthma, psoriasis, and rheumatoid arthritis.

Keywords: Euphorbiaceae, Castor oil, Immunomodulation, PBMN, Cytokines.

I. INTRODUCTION:
Ricinus communis or castor plant is a flowering plant species of the family Euphorbiaceae, which arises mostly in tropical countries¹, the plant exhibits noticeable antimicrobial activity, traditionally used to manage different ailments. Its parts such as leaves, roots, and seeds extracts exhibit therapeutic potentials including anti-inflammatory, hypoglycemic, and laxative effects². The various fractions of ethanolic, methanolic or hexane have been used for assessing the anti-inflammatory properties of the plant and this mainly due to the presence of important and effective compounds in the plant³. Identification of phytochemicals composition of crude aqueous extracts of leaves revealed the presence of tannins, steroids, and flavonoids⁴. While phenolic constituents include major antioxidant compounds such as quercetin, epicatechin, gallic acid, and ellagic acid⁵. The flavonoids interfere with aenormous regulatory pathways including cellular division and growth, transcription and genetic repairs, neuron signaling, stress and inflammation which may play a part in tumorigenesis⁶. Quercetin been reported to suppress tumor progression by inhibiting bio-chemical events. Also, quercetin can prevent the accumulation of recently produced protein called p53 without interfering with the mRNA levels in cancerous cell
Vero cells were regulatory cells isolated and cultivation were prepared using Soxhlet. Solutions were dried at 40°C. Peripheral blood was withdrawn from apparently healthy young donors, left for 30 minutes and allowed to be cooled to room temperature, then the blood was carefully added to 5 ml of density gradient medium (1.077 g/ml), ensure not mixing them, then supernatant was piped off. The monocytes will adhere to the surface and the lymphocytes remain in suspension. The monocytes can be subdivided into PBMNs (polymer-styrene tube, washed twice with phosphate buffer saline and centrifuged for 500 g for 10 minutes. The suspension was then used for separation of mononuclear cells through density gradient is the most widely used techniques for separation of mononuclear cells. Aqueous leaves extract, Ricinus communis leaves extract at concentrations of (500 and 125) µg/ml respectively, while the aqueous extract of leaves (1000, 500, 250, 125, 62.5, and 31.25) µg/ml and incubated for 24 hours at 37°C and 5% CO2. In this step, the monocytes will adhere to the surface and the lymphocytes remain in suspension. Vero cell line was kindly obtained from the Tissue Culture Lab. The cells are seeded in 96-well cell culture plate and incubated overnight to reach 80% confluence. Then, 200 µl of complete growth medium containing the aqueous or ethanolic extracts at final concentrations of (1000, 500, 250, 125, 62.5, and 31.25) µg/ml and incubated for 48 hours at 37°C and 5% CO2. Five replicates used for each concentration. Isolated PBMCs seeded immediately in 96 tissue culture plate. The cells treated with the same concentration mentioned above, then the plate incubated for 24 hours. At the end of the exposure period, the viability of the cells assessed by MTT cytotoxicity assay. The following cytokines were measured using ELISA kit: Interleukin-12 (IL-12), and Interleukin-10 (IL-10). The expression of IL-2, IL-13, IL-15, IL-17, and INF-γ secreted from PBMNs.

II. METHOD:
Separation of mononuclear cells through density gradient is the most widely used techniques for separation procedure that will ensure maximum lymphocytes recovery and minimum amount of granulocytes and erythrocytes contamination. Briefly, 5 ml of peripheral blood was withdrawn from apparently healthy young donors, left for 30 minutes and allowed to be cooled to room temperature, then the blood was carefully added to 5 ml of density gradient medium (1.077 g/ml), ensure not mixing them, then centrifuged for 30 minutes at 500 g. PBMCs layer appeared as the first cloudy band from top. PBMCs were seeded in 96-well cell culture plate and incubated overnight to reaches 80% confluence. Then, 200 µl of complete growth medium containing the aqueous or ethanolic extracts at final concentrations of (1000, 500, 250, 125, 62.5, and 31.25) µg/ml and incubated for 24 hours at 37°C and 5% CO2. Five replicates used for each concentration. Isolated PBMCs seeded immediately in 96 tissue culture plate. The cells treated with the same concentration mentioned above, then the plate incubated for 24 hours. At the end of the exposure period, the viability of the cells assessed by MTT cytotoxicity assay. For the evaluation of the immunomodulatory effects of Ricinus communis aqueous extract, PBMCs were seeded in 48 wells plate in five groups, the first group is the negative control (untreated cells), the second group were treated with (40 µl/ml) of PHA (phytohemagglutinin) mitogen and used as a positive control, the third and fourth groups were treated with Ricinus communis aqueous extract of leaves at concentrations of (500 and 125) µg/ml respectively, while the

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fifth and sixth groups were treated with a combination of PHA and Ricinus communis aqueous leaves extract at concentration 500 μg/ml and 125 μg/ml respectively. After 24 hours of incubation, the supernatant of each well taken for immunoassay by ELISA technique to evaluate the levels of IL4, IL10, IL17 and IFN-Gamma cytokines. All data were processed and analyzed using Microsoft Office Excel 2016 and Sigma plot version 12.5 software. ANOVA test used to assess significant differences among the means of the groups. P-values less than 0.05 considered statistically significant.

III. RESULTS:

Effect of Ricinus communis leaves extract on Vero cell line: Cells treated with aqueous and ethanolic extracts showed different responses (Fig. 1). Aqueous extracts showed less cytotoxic effect than ethanolic extract, the results showed a significant (P ≤ 0.001) decrease in the viability at the concentration of 1000 μg/ml only. While for ethanolic extract there was a significant (P ≤ 0.001) decrease in the viability of Vero cells at all concentrations used, in comparison with the control group (Fig. 1).

![Figure 1: Effects of aqueous and ethanolic leaves extracts of Ricinus communis on the viability of Vero cell line.](image)

Effect of aqueous and ethanolic extract of Ricinus communis leaves on isolated PBMNs

The aqueous extract, results showed a statistically significant (p < 0.05) increase in cells viability at a lower concentration (31.25 μg/ml) and a significant (p < 0.05) decrease in cells viability at the higher concentrations (1000 μg/ml and 500 μg/ml) compared to control group. While ethanolic extract showed no noticeable toxic effect on the cells (Fig. 2).

![Figure 2: The effects of Ricinus communis aqueous and ethanolic leaves extracts on the viability of the isolated peripheral blood lymphocytes.](image)

Immunomodulatory effects of Ricinus communis aqueous leaves extract

PHA (40 μg/ml) is a known mitogen, thus significantly induced the production of IL-17, IL-10, IL-4, and IFN-Gamma (Fig. 3). The plant extract showed significant suppression of IL-17 and IL-4 release at concentrations of
500µg/ml and both 500 and 125 µg/ml, respectively, in comparison to the control groups (Fig.3 A&C). R. communis extract caused a significant increase in IFN-Gamma at a concentration of 125 µg/ml in comparison to the control group (Fig.3 D). Plant extract showed no significant effect on IL-10 levels compared to the negative control group (Fig.3 B). On the other hand, plant combination with PHA diminished the mitogenic effect of PHA and reduces its induction of all cytokines assessed when compare with the PHA group.

Figure 3: Effects of aqueous leaves extract of Ricinus communis on IL-17 (A), IL-10 (B), IL-4 (C) and INF-γ (D) secreted from the isolated human peripheral lymphocytes.

IV. DISCUSSION:

Previous studies documented that Ricinus communis plant contain various chemical constituents, each with different biological effects. Phytochemical studies of R. communis leaves extract revealed the presence of high percentages of flavonoids, steroids, saponins, alkaloids, and glycosides. One of the common effective compound is the flavonoids family. The induction of apoptosis is one of the possible mechanisms behind the anti-proliferative effects of flavonoids. In vitro flavonoids capable of suppressing growth of a variety of cell lines mainly by cell-cycle arrest and induction of apoptosis. Flavonoids interfere, arrest cell- cycle at G2/M phases, and suppress expression of cyclin B in cancerous cells, the main reason for G2/M phases arrest is thought to be due to suppression of cyclin B protein, which is regulated, by transcriptional levels of cyclin B gene. The inhibition of angiogenesis in vitro is explained by inhibiting the release of vascular permeability factor VEGF which play an important role in angio-genesis and vasculo-genesis. Several human cell lines upon exposure to the leaf extracts showed cytotoxic effects in a dose dependent fashion, the cytotoxic effects of R. communis leaf extracts in different solvents including hexane, dichloromethane, acetone, and methanol at concentration range of 100-500µg/mL were investigated on human Caucasian skin fibroblast (Bud-8) cell line by using MTT assay, also identified by means of morphological examination, nuclear staining, and flow cytometric analysis of DNA content. Translocation of phosphatidyl-serine moiety to the outer surface of cell membrane leading to loss of mitochondrial membrane potential.

The phytochemicals found in leaves such as quercetin and kaempferol induced cytotoxicity in both SW480 and HCT-116 cells (type of colon cancer cell line) is likely to involve the alterations and modulation of the cell cycle progression by blocking the S-G0/G1 transition of cell cycling, and an inhibition of DNA synthesis that culminated in the loss of cell viability. Plant mitogens such as phytohemagglutinin (PHA) activate peripheral blood mononuclear cells that binds to the carbohydrate moiety on cell surface, provoking transformation of small lymphocytes into lymphoblasts, and subsequent cell proliferation and release of lymphokines. PHA is widely used in medical research as a triggering mitogen that stimulate cell division of peripheral blood monocytes and as an inducer of cytokines and interferons. The mechanism of activation of lymphocytes include two signaling pathways, the first depends on the myeloid differentiation factor, while the second depends on the Toll interleukin-1 receptor with subsequent activation of interferon beta. Interleukin-17 is an inflammatory mediator; it can induce granulocyte colony-stimulating factor production and other chemokines. IL-17 can eliminate bacteria and fungi throughout the infections by promote production of...
defending which act as antimicrobial peptides. During chronic infections and inflammations, such as allergies and rheumatoid arthritis, IL-17 plays an important role. Thus, inhibiting IL-17 may be useful in hindering chronic and autoimmune inflammations such as Psoriasis and multiple sclerosis25. Anthocyanins are water-soluble members of the flavonoid group, which resembles the most abundant content of the leaves. Anthocyanin suppress Th17 cells and inhibit the expression of proinflammatory cytokines including IL-17 by blocking the NF-κB pathway and inhibiting cyclooxygenase pathway 26. This may partially explain the suppressive effects of plant extract on IL-17 production even in the presence of the standard mitogen (PHA). When there is an antigen or mitogen, the naïve T cells normally activated and further differentiated into two types of effector cells called T-helper1 and T-helper 2. Th2 cells produce mainly IL-4 and IL-10, thus causing an increase in the levels of IL-10 produced by these lymphocytes in the presence of a mitogen 26. High levels of IL-10 can suppress pro-inflammatory cytokine production either directly by targeting immune effector cells or indirectly by modulate the function of immune system by prevent maturation of dendritic cells or macrophages, hence preventing antigen presentation and limit chemokine secretion capacity of the host. 28. IL-4 plays an important role in regulating the responses of lymphocytes, myeloid cells, and non-hematopoietic cells, hence the aqueous leaves extract may be useful in suppressing allergy, asthma and parasitic infections by suppressing the levels of this cytokines decrease differentiation of Naïve CD4 T cells into T-helper2 cells, inhibiting also the class switching of immunoglobulins in B cells, several drugs are now available as IL-4 antagonist for treatment of allergy and asthma. 29. Th-1 cells produce IFN-γ that enhance cell-mediated immunity. In addition, T-helper1 and T-helper2 and their secreted cytokines in specific clinical conditions can cross-regulate each other. Quercetin stimulates the production and gene expression of T-helper1 derived cytokine, such as IFN-γ. Also quercetin can stimulate IFN-γ favoring a Th-1-mediated immune reaction while suppressing T-helper-2 or humoral immune response. Hence, quercetin can cause an increase in lymphocyte with intra-cytoplasmic interferon gamma as well as production and release of IFN-γ. These two cytokines considered as the golden player role in the regulation our immune responses 30.

V. CONCLUSION:

The aqueous leaves extract has cytotoxic effects on Vero cells and PBMN only at the higher concentrations, while ethanolic leaves extract has cytotoxic effects on Vero cells at all concentration used. R. communis aqueous leaves extract showed a significant decrease in IL-17, IL-10, IL-4 and IFN-gamma. This suggests a potential use of this extract in autoimmune diseases such as asthma, psoriasis, and rheumatoid arthritis.

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