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Purified *Azadirachta indica* leaf arginase exhibits properties with potential tumor therapeutics: An Invitro Study

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Abstract

Except for a few key mechanisms, such as the continual arginine degradation by arginase, which is more often expressed in tumors than in normal cells, many advancements in understanding tumor immunity remain poorly understood. Thus, in order to comprehend *Azadirachta indica's* arginase function, as a possible therapy against tumor cells, a study to isolate, purify, and assess its arginase inhibitory characteristics was conducted.

Arginase isolation was done by subjecting the homogenate to 80% ammonium sulphate precipitation and dialysis respectively. The resultant solution was further purified using Biogel P-100.

Arginase from neem was greatly inhibited by salicylic acid than the oxalic acid, as there was drastic inhibition by ascorbic acid but mildly inhibited by citric acid. EDTA and phenyalanine demonstrated concentration- dependent inhibition properties on the enzyme

The overall results showed greater inhibition of arginase in the presence of these compounds, as this could suggest an improvement in the formulation of cancer therapy. Furthermore, the findings would be strongly recommended for future emerging research and drug development for cancer.

Keywords: Inhibitor, Neem, arginase, tumour-immunity

INTRODUCTION

Azardirachta indica (Neem) is a Meliaceae plant native to India and parts of Africa.[1] The plant's numerous biological properties have earned it the moniker "Village Pharmacy," as almost every part of the tree, including the bark, fruit, seeds, leaves, sap, and twigs, of multi-factorial pharmacological importance.

Arginase is a ureohydrolase enzyme. By converting arginine to urea and ornithine, the enzyme catalyzes the final reaction of the urea cycle, a series of biochemical reactions in mammals for ammonia detoxification.[2,3]

Mammalian arginase isozymes have been identified. Arginase I is primarily found in the hepatic cytoplasm, whereas Arginase II has been linked to the regulation of intracellular arginine/ornithine levels. This is found in the mitochondria of many tissues throughout the body, most notably the kidney and prostate, but less frequently in macrophages, lactating mammary glands, and the brain.[4] In the understanding of anti-cancer and biological properties of tumor therapeutic compounds, many major mechanisms have been proposed. Among these, the major mechanism of L-tryptophan and L-arginine catabolism by indoleamine 2,3-dioxygenase or tryptophan 2,3-dioxygenase, and arginase, which is constantly expressed in tumors, is still present. According to recent research, inhibiting these enzymes may improve anti-tumor immunotherapy potential.. As a result, the discovery of new arginase inhibitors is valued in the medicinal and pharmaceutical industries; thus, the current inhibitory study of purified *Azardirachta indica* arginase, because the threshold in the ammonia detoxification mechanism remains the function of arginase, and in the context of cellular ammonia regulation, this factor determines the amount of urea produced by mammals, as implicated in cancer. To evaluate the anti-tumor properties of Azardichta indica, the study purified arginase from its leaf and investigated the effects of some natural compounds on the enzyme.

MATERIALS AND METHODS

Plant

Azardichata indica (neem) leaf was obtained from a settlement (Olotoro Quarters), Oke-Ila, Ado-Ekiti, Southwestern Nigeria. The plant was immediately taken to the herbarium, for identification.

Chemicals

L-arginine, manganese chloride, CM-Sephadex C-50, phenyalanine, boric acid, sodium borate, EDTA, zinc sulphate, acetic acid, citric acid, lactic acid, ascorbic acid, oxalic acid and salicylic acid were obtained from Sigma Chemical Company, St Louis, USA. Remaining chemicals used were of analytical grade.

Preparation of *Azadirachta indica* Homogenate

Approximately 30 g of the neem leaf was sorted and homogenized with 0.004 M Tris buffer, pH 7.2. The mixture was cheese-clothed and centrifuged at 500*g (4 ^{0}C) for 1 h to achieve a clear supernatant, which was kept frozen for further use.

Arginase Activity Assay and Protein Determination

The enzyme assay was performed using method by[5] with minor modifications. In a final volume of 1 ml, a reaction

mixture containing 50 mM sodium carbonate buffer (pH 10), 1 mM $MnCl_2$, 0.33 M arginine solution, and an appropriate volume of enzyme preparation was added. The mixture was then incubated at 37 °C for 10 minutes before the reaction was stopped with the addition of 2.5 ml of Erhlich reagent. After 20 minutes, the absorbance at 450 nm was measured. The amount of urea produced was calculated using a standard urea curve prepared with varying urea concentrations. Arginase activity is defined as the amount of enzyme required to produce 1 mol of urea per minute. The protein concentration was determined using method by.[6]

Effects of Organic Acids on *Azadirachta indica* Arginase

To investigate the effect of organic acids on enzyme activity, 0.05 ml of the enzyme was incubated for 10 minutes with oxalic acid and salicylic caid at concentrations of 1, 5, and 10 mM.

Effects of Some Naturally Occurring Compounds on *Azadirachta indica* Arginase

Ascorbic acid and citric acid at concentrations of 1, 5, and 10 mM were added to 0.05 ml of the enzyme for 10 minutes to examine the impact of the natural acids on the enzyme activity.

Effects of Some Metallic Salts on *Azadirachta indica* Arginase

0.05 ml of the enzyme was incubated for 10 minutes with potassium chloride, zinc sulphate, ammonium sulphate, magnesium sulphate and sodium chloride at concentrations of 1, 5, and 10 mM each to examine the impact of these salts on enzyme activity.

Effects of EDTA and Phenylalanine on *Azadichta indica* Arginase

0.05 ml of the enzyme was incubated for 10 minutes with ethylene diamine tetraacetic acid and phenylalanine at concentrations of 1, 5, and 10 mM each to examine the impact of these compounds on enzyme activity.

RESULTS

Figure 1 displays the elution profile of the ammonium sulphate precipitated arginase isolated from the cane rat stomach using a Biogel P-100 column. Table 1 provides a summary of the arginase purification data, with a yield of 25% for the enzyme following ion exchange chromatography

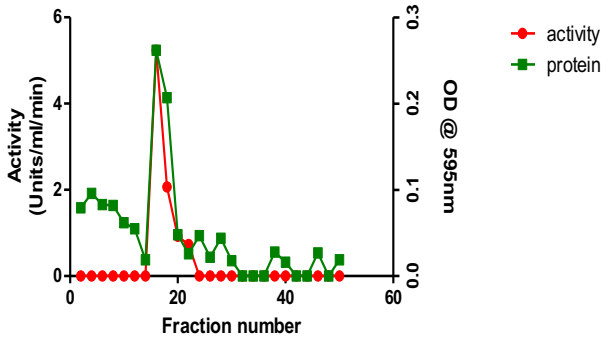

Figure 1: Elution Profile of *Azadichta indica* Arginase on Biogel P-100 Gel Filtration
Protein at 595 nm
Arginase at 450 nm

Table 1: *Azadirachta indica* Arginase Purification Table

Purification Step	Total Protein (mg)	Total Activity (Units/ml/min)	Specific Activity (U/mg)	Purification Fold	% Yield
Crude	234	120	0.51	1.00	100
80% Ammonium Sulphate Precipitation	98	70	0.71	1.40	58
Biogel P-100	50	30	0.6	1.20	25

Effects of Organic Acids on *Azadirachta indica* Arginase

Figure 2 showed a greater inhibition by salicylic acid than the oxalic acid

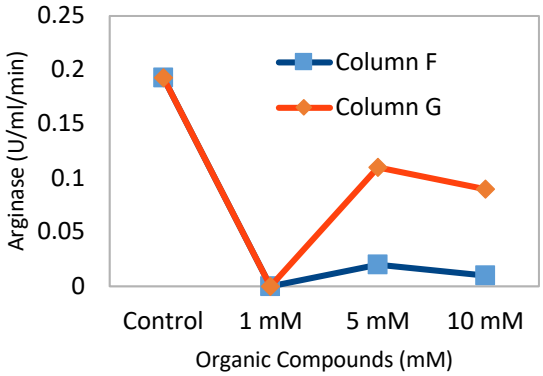

Figure 2: Effect of Salicylic Acid and Oxalic Acid on *Azardirachta indica* Arginase
Column F: Salicylic Acid (mM)
Column G: Oxalic Acid (mM)
Control: Arginase reaction without the organic compounds

Effects of Some Naturally Occurring Compounds on *Azadirachta indica* Arginase

The result showed the purified arginase was drastically inhibited by ascorbic acid but mildly inhibited by citric acid (Figure 3).

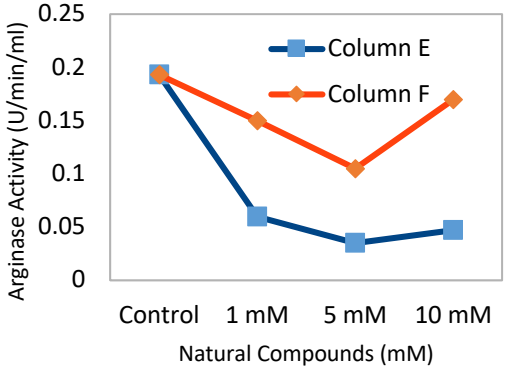

Figure 3: Effect of Natural Compounds on *Azadirachta indica* Arginase
Column E: Ascorbic Acid (mM)
Column F: Citric Acid (mM)
Control: Arginase reaction without the organic compounds

Effects of Some Metallic Salts on *Azadirachta indica* Arginase

Figure 4 showed varying inhibition effects on arginase purified from *Azardichta indica* leaf.

Effects of EDTA and Phenylalanine on *Azadirachta indica* Arginase

Effects of EDTA and phenyalanine demonstrated concentration- dependent inhibition properties on the enzyme (Figure 5).

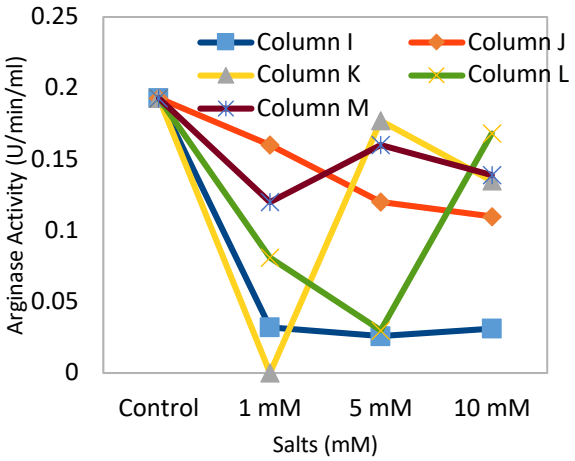

Figure 4: Effect of Salts on *Azadirachta indica* Arginase
Column I: Ammonium Sulphate (mM)
Column J: Magnessium Sulphate (mM)
Column K: Sodium Chloride (mM)
Column L: Potassium Chloride (mM)
Column M: Zinc Sulphate (mM)
Control: Arginase reaction without the Salt compounds

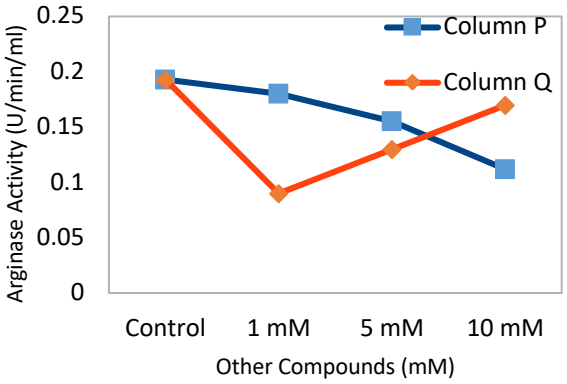

Figure 5: Effects of EDTA and Phenyalanine on *Azadirachta indica* Arginase
Column P: EDTA (mM)
Column Q: Phenyalanine (mM)
Control: Arginase reaction without the other compounds

DISCUSSION

Azardichta indica leaf arginase purification showed a specific activity of 0.6 U/mg of protein and a 25% yield. Purified arginase from plant leaves inhibited the reaction differently when it was exposed to different compounds, ranging from naturally occurring compounds to synthetic compounds, in order to study the interaction in the mimicry of a natural environment and in the production of drugs using specific chemicals. The study used organic compounds of salicylic acid and oxalic acid to exhibit inhibitory qualities, with salicylic acid having a more noticeable effect than oxalic acid, which is concentration dependant. The better inhibition effect could be as a result of the arginine substrate binding prevention posed by the chemical composition of the salicylic acid.[7] The oxalic acid which is known as a chelating agent could not have stopped the proceed of arginase reaction completely, owing to its structural classification, but could only induce partial inhibition mechanism, probably, due to its solubility.[8] Ascorbic acid is another compound that showed greater inhibition compared to citric acid. Though ascorbic acid is classified water soluble, this was not enough to stimulate the binding of the conventional arginine substrate, probably its structure stability could have been responsible for the inhibitory role or the binding distortion in the active site of the enzyme.[9]

Manganese acts as a cofactor, activator, and stabilizing component in arginase, a metalloenzyme. This requirement for a divalent cation has been documented for arginase function in both eukaryotes and prokaryotes.[5,10,11] The effects of salts (ammonium sulphate, magnesium sulphate, sodium chloride, potassium chloride, and zinc sulphate) on arginase activity were studied for this experiment, and it was discovered that the presence of ammonium sulphate had a significant inhibitory effect on the enzyme's activity at all concentrations. However, at 1 and 5 mM, potassium chloride had a diminishing effect. The enzyme activity was moderately inhibited by all other salts (magnesium chloride, sodium chloride, and zinc sulphate). This was in an agreement with[2], who reported the interaction of Na^+ on stomach arginase to be concentration dependent, with the lowest and highest inhibitions occurring at 5 and 0.1 mM, respectively. According to the same study, the interaction of manganese (II) chloride and mercury chloride exhibited increasing effects on enzyme activity, with the lowest and maximum inhibitions being at 10 and 0.1 mM, 0.5 and 0.1 mM, respectively. As a result, Mn^{2+} has been found to be a physiological activator of arginase.[2] [12] discovered that Mn^{2+}, Na^+, NH_4^+, and Hg^{2+} significantly increased grasshopper gut arginase activity, although Mn^{2+} and Zn^{2+} somewhat hindered the enzyme. Furthermore,[13] found that Zn^{2+} and Mg^{2+} significantly increased the activity of *Periplaneta americana* gut arginase while Sn^{2+}, Hg^{2+}, and Ni^{2+} inhibited it. [2] reported that the interaction of urea and EDTA has a concentration dependent influence on enzyme activity, with the lowest and highest inhibitions occurring at 1 and 2 mM and 5 and 1 mM, respectively. EDTA is a chelating agent that has been found to totally block arginase.[2]

[13] reported that EDTA substantially suppressed grasshopper arginase. [14] observed a significant suppression of Saccharomyces cerevisiae arginase by EDTA. [15] reported that EDTA, citrate, and urea substantially suppress arginase activity in the hepatopancreas of gigantic freshwater prawns (*Macrobrachium rosenbergii*). In this investigation, both EDTA and phenyalanine inhibited the enzyme.

CONCLUSION

The overall results showed greater inhibition of arginase in the presence of these compounds, as this could suggest an improvement in the formulation of cancer therapy. Furthermore, the findings would be strongly recommended to provide biotechnology insight and future emerging research in the development of drugs for cancer.

Conflict of Interest

The authors declare no "conflict of interest"

Data Availability Statement

All data of the study will be made available by the corresponding author on request

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