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Annona Muricata reduced Hepatocytes Differentiation Caused by Cycas revoluta in Wistar Rats

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Abstract: Our study aims to evaluate the action of *Annona muricata* ethyl extract in the traditional treatment of liver cancer in Wistar rats. This is an analytical, prospective and control study that took place at the Laboratory of Biomembrans and Cell Signaling of the University of Abomey Calavi. The objective of this study is to verify the effects of the ethyl extracts of *Annona muricata* leaves on the differentiation of hepatocytes in the Wistar rat. For this, an ethyl extraction was carried out using the dried leaf powder of *Annona muricata* and ethanol at 90°, as well as the extraction of the leaves of *Cycas revoluta*. *Cycas revoluta*-induced differentiation-lowering activity was assessed by assaying the total protein and alkaline phosphatase (PAL) parameters on a Wistar rat liver mash supernatant in which hepatocyte differentiation had previously been induced by exposure to the powder and ethyl extract of *Cycas revoluta* by gavage. Our sample included 3 batches of Wistar rats. Batch 1 was the control batch, batches 2 and 3, respectively exposed to the powder and the extract of *Cycas revoluta* for 2 weeks, then subjected to gavage 100 mg / kg of extracts of *Annona muricata* for 2 more weeks. At the end of our study it was revealed that the activity of PAL and the total protein content which was high following exposure to *Cycas revoluta*, was significantly reduced after the treatment with ethyl extracts of *Annona muricata* leaves. In addition, it was also the ratio PAL / total protein that accounts for the stage of differentiation, which was increasing under the effect of *Cycas revoluta*, decreased after administration of the ethyl extracts of the leaves of *Annona muricata*. These results make it possible to demonstrate the action of *Annona muricata* extracts on the differentiation of Wistar rat liver cells induced by a carcinogenic substance such as powder and extract of *Cycas revoluta*.

Keywords – *Annona muricata*, *Cycas revoluta*, hepatocytes, extract, differentiation

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I. Introduction

Non communicable diseases represent an increasing burden on health systems, due to a double demographic and epidemiological transition, in this case in the developing countries. They include cardiovascular diseases, type 2 diabetes, cancer ... and are rising all over the world, including the countries of the south, in terms of incidence and mortality. Cancers are a major cause of morbidity and mortality in the world. According to estimates by the World Health Organization (WHO), they accounted for 7.6 million deaths in 2008, accounting for 13% of total mortality ⁽¹⁾. Many environmental factors related to lifestyle and industrialization are blamed for the increased incidence of cancers. Epidemiological data predict a steady increase in cancer mortality. By 2030, 13 to 17 million people will die of cancer each year ⁽²⁾. In Africa, 600,000 cases of cancer are reported each year and 500,000 people die, according to WHO. Indeed, cancer is a group of various diseases, characterized by abnormal cell proliferation, invading and destroying the surrounding tissues, and can spread to give rise to metastases either in the organ of origin or in different organs ⁽³⁾. This dysfunction could be due to the intervention of exogenous factors. These factors include alcohol and tobacco consumption, low consumption of fruits and vegetables, and chronic viral infections. ⁽⁴⁾ To this end, the most frequently diagnosed cancers in the world are those of lung, liver, breast and colorectal cancer ⁽⁵⁾. The liver cancer, that we study, usually occurs as a result of liver disease. ⁽⁶⁾ According to the WHO 70% of all liver cancers deaths occurred in developing countries that focus mainly in Africa ⁽⁷⁾. Indeed, Africa has many plants with various therapeutic virtues. These plants have the ability to produce very diverse natural substances. They are recognized as a source of drugs ⁽⁸⁾⁽⁹⁾. This is why medicinal plants are used in the management of chronic diseases such as liver cancer in low-income countries, particularly in Africa ⁽¹⁰⁾. It is for this purpose that the extracts of the leaves of *Annona muricata* are used in the traditional pharmacopeia to stop the proliferation of the cells of the liver. Our work will focus on the effects of *Annona muricata* on the differentiation of hepatocytes.

II. General information on *Annona muricata* and *Cycas revoluta*

❖ Biology of *Annona muricata*

To counteract the side effects of anti-cancer treatments, scientific research is carried out on several plants, such as *Annona muricata*, used in the traditional African pharmacopoeia ⁽¹¹⁾, especially in Benin. Originally from South America, corossol is also grown in other tropical regions. Its tree called « Guanabana » or « Graviola » offers exquisite fruits and leaves that would destroy many cancer cells. According to research conducted at the American University of Purdue, leaves of the sapling tree possess properties that destroy cancer cells ⁽¹²⁾. The hornbill is a shrub or small tree 3 to 10 m high. The leaves, of a brilliant green, are oblong-lanceolate, of 10-17 × 2-7 cm, the young with ferruginous pubescence below. The flowers appear on large pedicels (15-20 mm long) opposite the leaves. The 6 petals are yellow, fleshy and thick. The 3 external petals are largely oval at the edges, without being superimposed. It blooms all year round. It tolerates poor soils but cannot withstand low temperatures. The soursop, fruit of the hornbill is up to 30 cm long and can weigh up to 4 or 5 kg. Its external aspect is of a dark green, its bark pierced with thorns and its white pulpy flesh with black seeds. In Benin, we find the sourscher usually in the south. Being a fruit tree, domesticated, it is often grown in urban areas.



Figure 1. Fruits and leaves of *Annona muricata*

❖ Biology of *Cycas revoluta*

The genus *Cycas* contains about twenty species. This tree, similar to a dwarf palm, originating in Japan, reaches a height of 4m for a width of 3m. It has very slow growth on a robust trunk and an evergreen foliage, dark green, arranged in rosette. It is a dioecious plant whose male or female inflorescences are of no decorative interest. Rustic down to -10 ° C foliage is deteriorated from -3 ° C. It is a toxic plant. It synthesizes a very toxic glucoside for herbivores (cyclosin) and a neurotoxic amino acid, beta-Nmethylamino-L-alanine. Twelve hours after an animal ingests leaves of the tree, the animal begins to vomit, has diarrhea, nose bleeds and other symptoms that can lead to death. ⁽¹³⁾



Figure 2. Cycas revolute

III. Materials and Methods

❖ Preparation of extracts from leaves of *Annona muricata*

50 g of *Annona muricata* leaf powder weighed using a Sartorius® analytical balance were macerated in 500mL of ethanol for 72 hours with stirring. Then, the macerate is filtered through the hydrophilic fiber cotton. The filtrate obtained is evaporated with the aid of the ROTAVAPOR evaporator at 40 ° C. The recovered extracts were placed in an oven at 45 ° C. for drying. The dry extracts were scraped with the stainless steel spatula and then stored in glass bottles previously labeled. These extracts will be used to prepare the concentration ranges tested. The yield is determined by the ratio of the weight of the dry extract after evaporation to the weight of the dry vegetable material used for the extraction multiplied by 100⁽¹⁴⁾.

❖ Verification that *Cycas revoluta* stimulate differentiation of liver cells

Within context of this work, we have used 3 batch of 3 rats heaving approximately 150g (males and females). This rats has been submitted to the following treatment by gavage:

Batch	Treatment during the 2 first weeks	Treatment during the 2 last weeks
1 (control)	Distilled water	Distilled water
2	Usual granule + 5% <i>Cycas revoluta</i> powder + 10mg/kg <i>Cycas revoluta</i> powder diluted in drink water	100mg/kg of <i>Annona muricata</i> extract
3	100mg/kg of <i>Cycas revoluta</i> extract	100mg/kg of <i>Annona muricata</i> extract

The 15th day, one rat of each batch has been sacrificed, then dissected. After dissection, 0,5g of liver has been taken and crushed with acid sand and 5ml of physiological water. The mash liver has been centrifuged at 6000 trs/min for 5 minutes. After this, the total proteins and the alkaline phosphatase were then assayed on the resulting supernatant.

It is important to remember that *Cycas revoluta* extracts used in this experimental stage has been carried out with the same ethylic extraction process like as *Annona muricata* extraction.

❖ Pharmacological activity of the ethyl extracts of the leaves of *Annona muricata*

After, verification of hepatic cells differentiation induced by *Cycas revoluta*, the gavage continues with remaining rats. After the 4 weeks of gavage, the rats were sacrificed. Their livers were taken. Then, 0,5g of each organ was cut and crushed with acid sand and 5ml of physiological water. The mash liver was centrifuged; and biochemist parameters this are the total proteins and the alkaline phosphatase were then assayed on the

resulting supernatant in order to observe the impact of *Annona muricata* ethylic extract on the hepatic cells differentiation induce by *Cycas revoluta*.

- **Determination of biochemical parameters**

- **Total proteins**

- ✓ **Principle**

The total protein assay was carried out by the colorimetric method described by Gornall et al in 1949. The peptide bonds of the proteins react with cupric (Cu^{2+}) ions in alkaline solution to form a colored complex whose absorbance, proportional to the concentration in proteins in the specimen, is measured at 550 nm. The Biuret reagent contains sodium potassium tartrate which complexes cupric ions (Cu^{2+}) and maintains their solubility in alkaline solution. Mix. Leave for 10 minutes at a temperature of 20°C to 25°C . Read absorbance at 550nm (530-570) against reagent blank The calculation is made according to the following rule: (Abs. Dosage / Abs. Stallion) \times concentration of the standard.

- **Alkaline phosphatase**

- ✓ **Principle**

Optimized method based on the recommendations of the DGKC (Food Society of Clinical Chemistry, 1972) and the SCE (Scandinavian Society of Clinical Chemistry). In an alkaline medium, alkaline phosphatases catalyze the hydrolysis of p-nitrophenylphosphate to p-nitrophenol and phosphate. The rate of occurrence of p-nitrophenol, followed by the change in absorbance at 405 nm, is proportional

- ❖ **Statistical analysis**

Microsoft Excel (version 2010) software, has been used to data compiling, and data's variance analysis. Finally, an averages structuring was doing, to permit to compare the extracts impacts in order to identify, the extracts which have a significant impact by Student Newman and Keuls (SNK) test at the break-even line of 5%.

IV. Results

- ❖ **Verification that *Cycas revoluta* and differentiation of liver cells**

The results obtained from the comparison of the PAL level and total proteins, according to the type of treatment received by the rats, show a rather significant influence of the exposure of the rats to the powder or the extract of *Cycas revoluta* as regards the PAL rate. This results are justifying by a variance study with the student SNK test that gives us ($P \leq 0.05$). As for the total protein level, there is a slight non-significant variation with ($P \geq 0.05$).

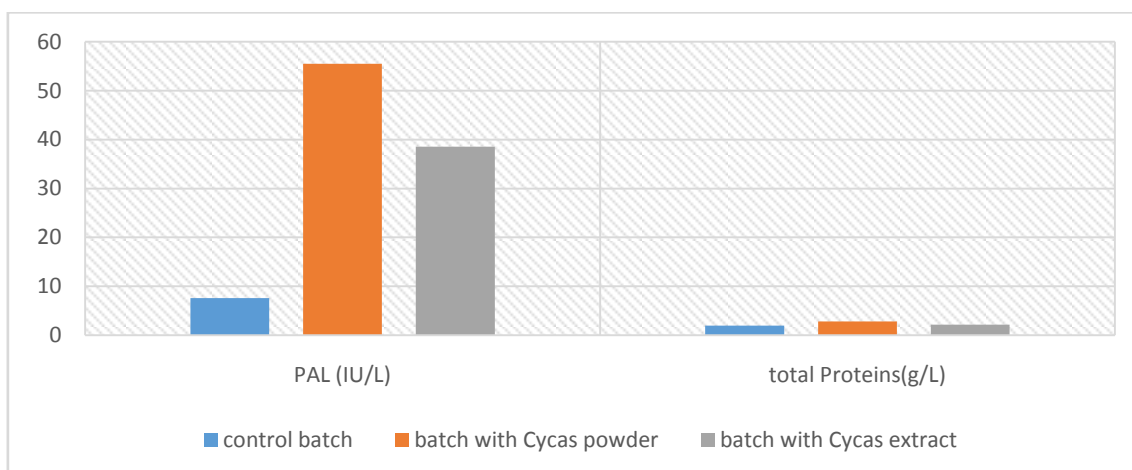


Figure 3. Total proteins and PAL tissue rates variations according to diet containing and not *Cycas revoluta*.

The comparison of the ratio PAL / total proteins according to the exposure or not of the rats to the powder or the extract of *Cycas revoluta*, shows us a significant increase ($P \leq 0.05$) of this ratio in the treated *Cycas revoluta* under its 2 forms, which evokes an increase in the process of cellular differentiation of hepatocytes.

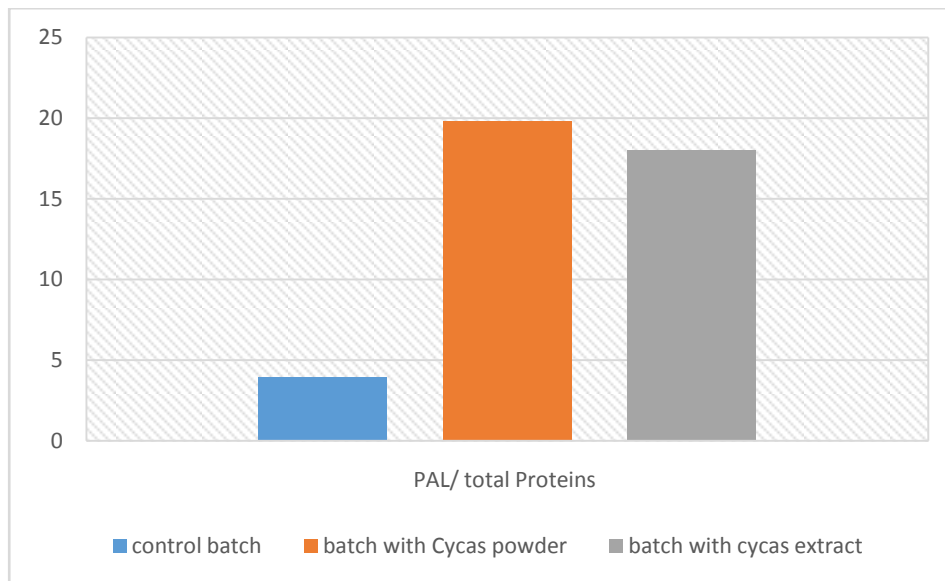


Figure 4. Variations of PAL / total proteins tissue ratio according to diet containing and not Cycas revoluta.

❖ **Pharmacological activity of the ethyl extracts of the leaves of *Annona muricata***

The results obtained from the comparison of the PAL level and total proteins, according to the type of treatment received by the rats, show a rather significant influence of the administration of the ethyl extract of *Annona muricata* leaves on the rats previously treated with *Cycas revoluta* by the PAL rate side. Indeed, the rate of PAL which was increase under the action of *Cycas*, falls after the administration of the extracts of *Annona muricata*. This results are justifying by a variance study with the student SNK test that gives us ($P \leq 0.05$). As for the total protein level, there is a slight non-significant variation with ($P \geq 0.05$).

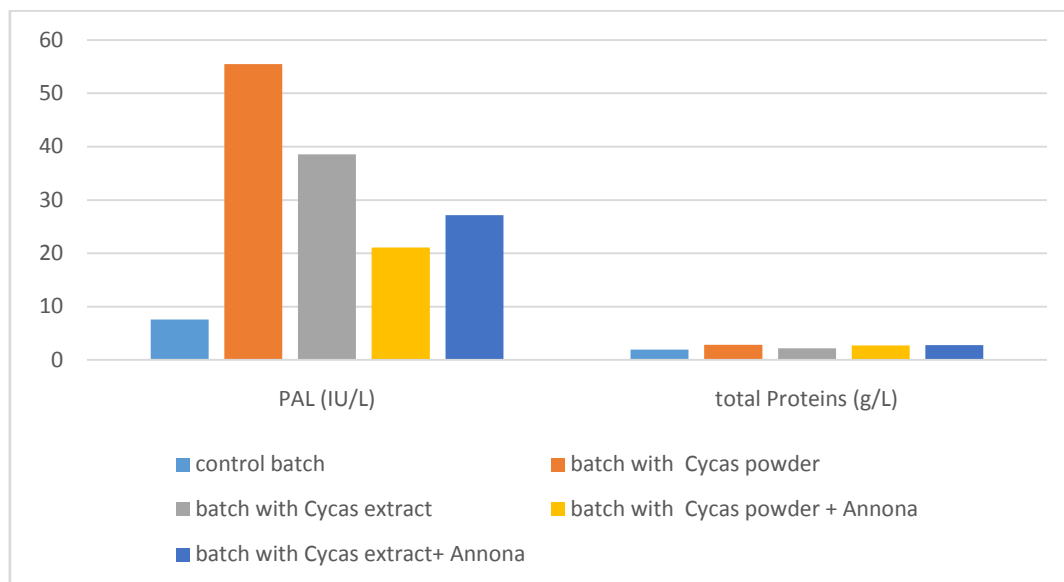


Figure 5. Total proteins and PAL tissue rates variations according to diet containing and not Cycas revoluta, and treatment with ethylic extract of *Annona muricata*

Following the administration of the *Annona muricata* extract, there was a significant fall ($P \leq 0.05$) in the PAL / total protein ratio, which was previously increased by *Cycas*. This result evokes a decrease in the differentiation process induced by *Cycas revoluta*, by the action of *Annona muricata* extracts.

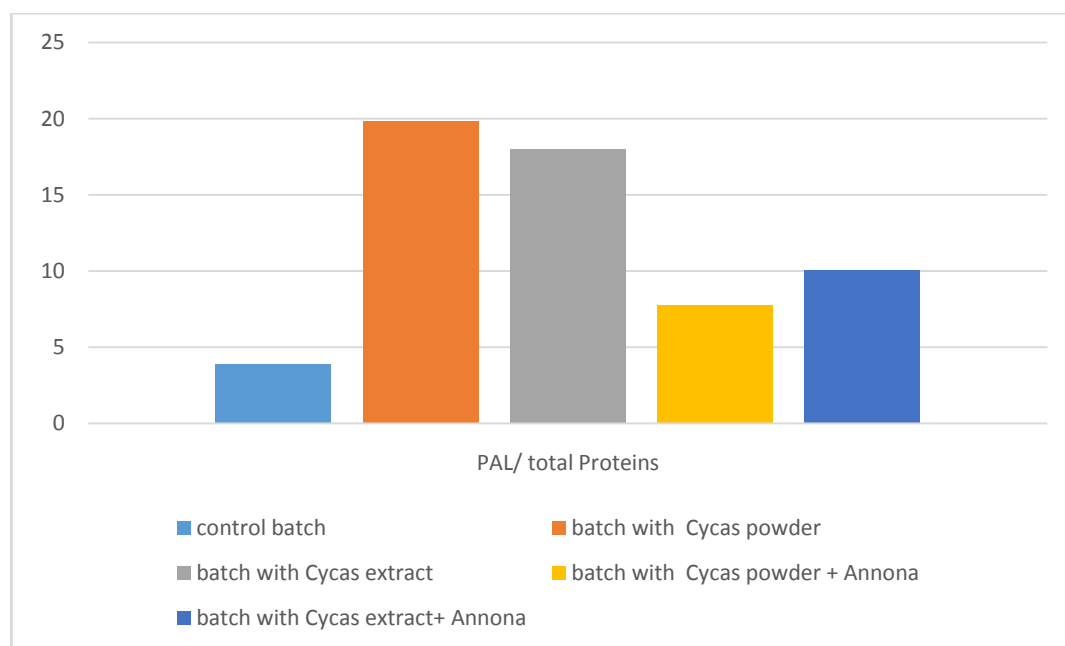


Figure 6. Variations of PAL / total proteins tissue ratio according to diet containing and not Cycas revoluta, and treatment with ethylic extract of Annona muricata

V. Discussion

According to the WHO in 2006, cancer is a term used to refer to autonomic and anarchic malignant proliferation of cells. They are a major cause of morbidity and mortality in the world. In Africa, 600,000 cases of cancer are reported each year and 500,000 people die, according to WHO. This may be due to the high cost of conventional treatment methods such as chemotherapy, radiotherapy, and surgery⁽¹⁵⁾. To remedy this, they therefore resort to traditional medicine based on the use of medicinal plants. In an extended study the anticancer properties of 187 plants were evaluated⁽¹⁶⁾, including *Annona muricata*. Many scientific studies have showed that *Annona muricata* has some anti-cancer activities. It is in this perspective that we used the leaves of *Annona muricata*. At the end of our study, we carried out an ethyl extraction using the powder of the leaves of *Annona muricata* and 90 ° ethanol. We obtained a yield of 10.5%, which we judge of means compared to the yield obtained by Eka Prasati et al., 2012, which is 14.86%. This difference could be explained by the different types of filters used in our experimentations. We also carried out an ethyl extraction of the leaves of *Cycas revoluta*. We had an average return of 10.2%. *Cycas revoluta* would contain Cycasin (methyl azoxymethanol) which is known to induce cancer. Since researchers have become aware of the effective carcinogenic properties of methyl azoxymethanol, these agents have been used to create reliable cancer animal models. This is why we used the powders and extracts from the leaves of *Cycas revoluta*. We therefore first checked the carcinogenicity of the powder and the extract of the leaves of *Cycas revoluta* on the proliferation and differentiation of cells of liver, because many types of liver cancers are differentiated.⁽¹⁷⁾

We therefore first verified the carcinogenicity of the powder and leaf extract of *Cycas revoluta*, which could be characterized by the proliferation and differentiation of liver cells. For this, the rats received a diet containing either the *Cycas* extract or the *Cycas* powder; then the total protein and PAL parameters were assayed. It should be noted that the PAL parameter was chosen because it is a cellular enzyme found at different levels of the cell, and that its rate reflects the state of the tissue, because an increase in this rate would be a sign of tissue pain.

At the end of our verification test, there was a slight increase in total protein and a significant increase ($P \leq 0.05$) in PAL levels in rats exposed to *Cycas revoluta* (powder and extracts). These results are consistent with those obtained by Okolie et al., In 2013 who used *Cycas circinalis* powder (a plant of the same family as *Cycas revoluta*) to induce colorectal cancer in wistar rats, states that *Cycas* stimulates increase in total protein and cellular enzymes. The total proteins and the PAL (cellular enzymes) being derived from the transcription of genes followed by the translation process, the increase of their levels at the tissue level, would suggest a stimulation of this transcription, which evokes a cellular proliferation, sign may suggest carcinogenesis.

Kouadio et al. (2006) point out that the PAL / total protein ratio accounts for the stage of differentiation. Our results show a PAL / total protein ratio significantly ($P \leq 0.05$) higher in the test rats compared to controls. These results, therefore, suggest that *Cycas revoluta* also stimulates differentiation of hepatic cells. The second phase of our tests concerned the study of anti-differentiation activity of the ethyl

extracts of *Annona muricata* leaves on hepatic cells of wistar rats previously exposed either to the powder or to the extract from *Cycas revoluta*. Our results show that after the administration of *Annona muricata* leaf extracts, there was a slight decrease in the total protein level and a highly significant decrease ($P \leq 0.01$) in the PAL level compared to rats exposed only to *Cycas revoluta*. In addition, the PAL / total protein ratio of the rats receiving the *Cycas* extracts and the *Annona muricata* extracts decreased considerably, as did the PAL / total protein ratio of those receiving the *Cycas* powder and *Annona muricata* leaf extracts. These results are consistent with those of Okolie et al., 2013, which showed anti-cancer activity of the ethyl extracts of *Annona muricata* leaves through anti-proliferative pharmacological properties on cancer cells. From our results, it follows that the ethyl extracts of leaves of *Annona muricata* could possess anti-differentiation properties on liver cells of the Wistar rat.

VI. Conclusion

The results of our study show that the powder and extracts of *Cycas revoluta* significantly stimulate the differentiation of the liver cells of the Wistar rat, and the extracts of the leaves of *Annona muricata* diminishes the differentiation process, which is an important stage of liver cancer genesis. These results have in many respects promoters for the fight against liver cancer suggest research on the mechanisms of action of these extracts.

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