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APOPTOTIC ACTION OF ETHANOLIC EXTRACTS OF THE LEAVES OF HEXALOBUS MONOPETALUS (ANNONACEAE) ON CERVICAL CANCER IN WISTAR RATS

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Keywords: *Hexalobus monopetalus, apoptosis, cancer, cervix.*

Abstract

Across the world, the search for new anti-cancer molecules remains one of the main concerns of oncology researchers. Also, plants have been at the origin of many active molecules having shown their effectiveness in the treatment of different cancers (MERGHOUB N., 2011).

In Benin, traditional medicine, rich and diversified, is an important source for the screening of new therapeutic molecules.

The aim of our study is to determine the apoptotic action of the ethyl extract of the leaves of Hexalobus monopetalus on cervical cancer in wistar rats. The total proteins were assayed to the spectrophotometer in the blood after induction of cervical cancer with Cycas revoluta in three batches of three rats each. Once the cancer is induced, the dose of 2.5 mg / kg body weight of the ethyl extract of the leaves of Hexalobus monopetalus diluted in 1 ml of 33% ethanol is administered to three batches of two rats each. The biochemical parameters of total proteins and alkaline phosphatase were measured on the tissues of the cervix.

It emerges from our work that Cycas revoluta has a proliferative action on the tissues of the cervix. Also, the ethyl extract of the leaves of Hexalobus monopetalus increases the level of total proteins and lowers the alkaline phosphatase level on cervical tissues. Hexalobus monopetalus would have an antiproliferative action on cancer cells of the cervix.

Introduction

Across the world, cervical cancer is a major public health problem. Cervical cancer causes about 200 000 deaths annually and 500 000 new cases each year, with 85% occurring among women in developing countries (POINTREAU Y. et al., 2010) Incidence and mortality. Cervical cancer, considered to be a sexually transmitted disease, is closely linked to infection with certain viruses called Papilloma Human Virus (HPV). These oncogenic viruses contribute to the immortalisation of the infected cells. The transforming power of these HPVs was attributed to the viral proteins E6 and E7 (MUNGER K. et al., 2004).

Cancers develop from abnormal cells that multiply in an uncontrolled way to the detriment of the body. Thus, apoptosis occurs in many physiological processes such as embryonic development, proliferation / homeostasis, differentiation, regulation and functioning of the immune system. Apoptosis is the precise orchestration that the body uses to get rid of unusable, unwanted or potentially harmful cells. Damaged or unwanted cells are eliminated by neighboring cells without causing inflammation due to leakage of cellular content into the cellular environment. Anomalies in the regulation of apoptosis contribute to various pathological conditions, including cancer. Cancer occurs when the balance between proliferation and cell death is disrupted by increased cellular proliferation or by decreased or deficient apoptosis (JIM, 2010). The mutation of certain genes is at the origin of their appearance (PATRICIA P., CHRISTINE H., 2013).

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Plants, marine organisms and micro-organisms constitute an important source of active substances having an important role in medicine. Many anti-cancer drugs of natural origin or derived from natural compounds are currently used in cancer chemotherapy. The exploitation of traditional pharmacopoeias, in particular the use of plants known for centuries for their medicinal value, has led to the introduction of a very large number of highly effective anti-cancer drugs (MERGHOUB N., 2011) .

Traditional Beninese medicine is therefore rich and diversified, which is an important asset and an inexhaustible source in the screening of new therapeutic molecules. Benin is a geographical unit whose characteristics offer a varied range of bioclimates allowing the installation of a very rich flora. In addition to this particularly favorable and promising context, this country has an ancestral know-how that has been preserved over the centuries: herbal medicines, their use for flavoring and preserving food, and Extraction of aromatic principles intended for family or commercial use (MERGHOUB N., 2011).

It was after a failure or a lack of costly hospital medicine, modeled on that of the rich countries, that WHO encouraged Third World countries to give a large part to their traditional pharmacopoeia (ADJANOHOUN et al . ,nineteen eighty one).

Hexalobus monopetalus, one of the six species of the genus Hexalobus of the Annonaceae family, is native to tropical Africa. It is found in countries such as Benin, Tanzania, Ivory Coast, Senegal. Its vegetative organs are used in the treatment of headache, diabetes (AKOUEGNINOU et al., 2006), candidiasis (HAMISI et al., 2014), etc. In the literature, many studies have not addressed fundamental studies on the leaves of this plant. It is thus that we have proposed to study its anti-carcinogenic activity through this work which we call: "Apoptotic action of the ethanolic extracts of the leaves of Hexalobus monopetalus (Annonaceae) on cervical cancer in rats Wistar. "

Materials and methods

Material Plant

The plant material consists of the leaves of Hexalobus monopetalus and Cycas revoluta. The leaves of Hexalobus monopetalus, were harvested in September 2014 to kouandé by the team of the Laboratory of Biomembranes and Cell Signaling then authenticated by the herbarium of the University of Abomey-Calavi. The leaves were dried in an oven at a temperature of 55 ° C. for at least 4 hours. These dried leaves were finely ground. The powder obtained is then stored in glass jars in order to avoid external contamination. It is this conditioned powder which has served us as plant material for our study. Some samples of Cycas revoluta are harvested in the geographical area of the University of Abomey-Calavi and authenticated by the herbarium of the University of Abomey-Calavi. The leaves are removed. The samples are dried away from light and moisture at the Biomembrane Laboratory and cell signaling for 2 months, then finely ground and placed in glass jars to avoid any external contamination.

Animal equipment

We have three (03) batches of six (06) female wistar rats reaching puberty (at least 2 months). The animals are subjected to daily gavage for 28 days.

Methods

II-2-2-1-Preparation of the ethanol extracts of the leaves of Hexalobus monopetalus and the leaves of cycas revoluta
To obtain the ethanolic extracts of these various sheets, 100 g of powder obtained, weighed using a Sartorius® analytical balance, are macerated in 1000 ml of ethanol with continuous stirring for 72 hours. Then, the macerate is filtered through the hydrophilic fiber cotton. The filtrate obtained is evaporated at 50 ° C. for Hexalobus monopetalus and at 40 ° C. for Cycas revoluta using the Rotavapor® rotary evaporator. The paste deposited at the bottom of the evaporator flask is recovered in jars and dried in an oven at 45 ° C. After drying completely, the dry

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extracts are placed on the bottom of the jars, scraped with a stainless steel spatula, crushed in porcelain mortar and then kept 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 matter used for the extraction multiplied by 100 (MEDANE. 2012).
Rd% = (m1x100) / m2
M1: mass of the dry extract after evaporation
M2: the mass of the dry plant material

Gavage of rats
For our work, we have three (03) lots of six (06) female rats each weighing 200g of body weight on average. Subcutaneous gavage is carried out for fourteen (14) days with an ethanolic extract of the leaves of Cycas revoluta (lot 2) and with the powder of Cycas revoluta (lot 3)

Gavage of rats with Cycas revolute

Table III: Distribution of rats per batch and per dose of Cycas revoluta

Lot 1	Lot 2	Lot 3
Granulated + distilled water (without Cycas revoluta)	Granuleated + 100mg / kg body weight of the ethanolic extract of Cycas revoluta dissolved in 1ml of distilled water	Mixed granulated of 5% Cycas revoluta powder + 10mg / kg body weight of Cycas revoluta powder dissolved in 1ml of distilled water

Verification of carcinogenic effect by Cycas revoluta: Determination of biochemical parameters

The total proteins are assayed in the blood on Day 0 (D0), Day 7 (D7) and Day 14 (D14), respectively, in the three batches of rats.

On the 14th day, two rats of each batch are sacrificed, dissected and the cervix is extracted.

- On the one hand, the removed cervix is rinsed in physiological water solution and then stored in the PBS solution for five days in the refrigerator, after which total proteins and PAL have been assayed In the PBS solution (liquid in which the cervix stayed).
- On the other hand, the cervix was ground in a porcelain mortar. The ground material obtained was centrifuged at 6000 rpm for 5 minutes. The total proteins and PAL were re-assayed in the supernatant obtained after centrifugation of the cervix.

Gavage of the rats with the ethanolic extract of the leaves of Hexalobus monopetalus

The previous batches were conserved and subcutaneous gavage of the rats remaining for 14 days with the ethanol extract of the leaves of Hexalobus monopetalus

TABLE IV Distribution of rats per batch and per dose of Hexalobus monopetalus

Lot 1	Lot 2	Lot 3
Granulated + distilled water (without Hexalobus monopetalus)	Granulated + 2.5 mg / kg body weight of ethanolic extract of H. Monopetalus dissolved in 1 ml of ethanol at 33%	Granulated + 2.5 mg / kg body weight of ethanolic extract of H. Monopetalus dissolved in 1 ml of ethanol at 33%

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Verification of the antiproliferative effect of the extract of Hexalobus monopetalus: determination of biochemical parameters

The total proteins are assayed in the blood on the 14th day (D14), the 21st day (D21) and the 28th day (D28) in the three batches of rats.

On the 28th day, all the rats of the three batches are sacrificed, dissected and the cervix is extracted.

- On the one hand, the removed cervix is rinsed in physiological water solution and then stored in the PBS solution for five days in the refrigerator, after which total proteins and PAL have been assayed In the PBS solution (liquid in which the cervix stayed).
- On the other hand, the cervix was ground in a porcelain mortar. The ground material obtained was centrifuged at 6000 rpm for 5 minutes. Total proteins and PAL were re-assayed in the supernatant obtained II -2-2-2-5- Blood sample

The blood sample is taken according to the experimental protocol modified byDESCARTES (2002). Puncture of the retro-orbital sinus was performed. The animal is held with one hand in lateral decubitus, and held by the skin of the neck. The pressure of the thumb on the neck, behind the angle of the jaw, allows compression of the jugular vein, and therefore venous stasis towards the head, favoring the filling of the retro-orbital sinus. By making a slight traction on the upper eyelid with the index finger, we create an exophthalmos facilitating the taking of blood by means of tube with hematocrit not heparinized. The end of the tube is slowly introduced into the lateral angle of the eye. Progression through the tissues is facilitated by printing a small pipette rotation. As soon as the venous plexus is reached, the blood springs into the perorbital space and ascends by capillary action in the tube. The volume of blood collected is 0.5 to 2 ml. Before the tube is removed, the compression is released and the bleeding ceases spontaneously when the ocular pressure normalizes. The recovered blood is used for the determination of the various biochemical parameters.

- after centrifugation of the cervix of the cervix.

Results and discussion

Results

Extraction yield

The yield is calculated by the formula:

Rd% = (m1x100) / m2

M1: mass of the dry extract after evaporation

M2: the mass of the dry plant material

TABLE VII: Yield of the ethanolic extract of the leaves of Hexalobus monopetalus

Color	Mass (g)	Yield Percent (%)
Brown	11,08	**R=11,08×100/100 R=11,08**

The yield of 11.08% obtained for extraction is low.

TABLE VIII: Yield of the ethanolic extract of the leaves of Cycas revoluta

Color	Mass(g)	Percent(%)
Dark green	**10,2**	**R=10,2×100/100 R=10,2**

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Results of biochemical tests

Verification of the carcinogenic effect of Cycas revoluta

On day 7, there was no variation in the level of total proteins in the blood. On the other hand, on the 14th day, this rate increased.Cycas does not affect the level of total proteins in the blood. However, there is an increase in this rate over time but this is not significant

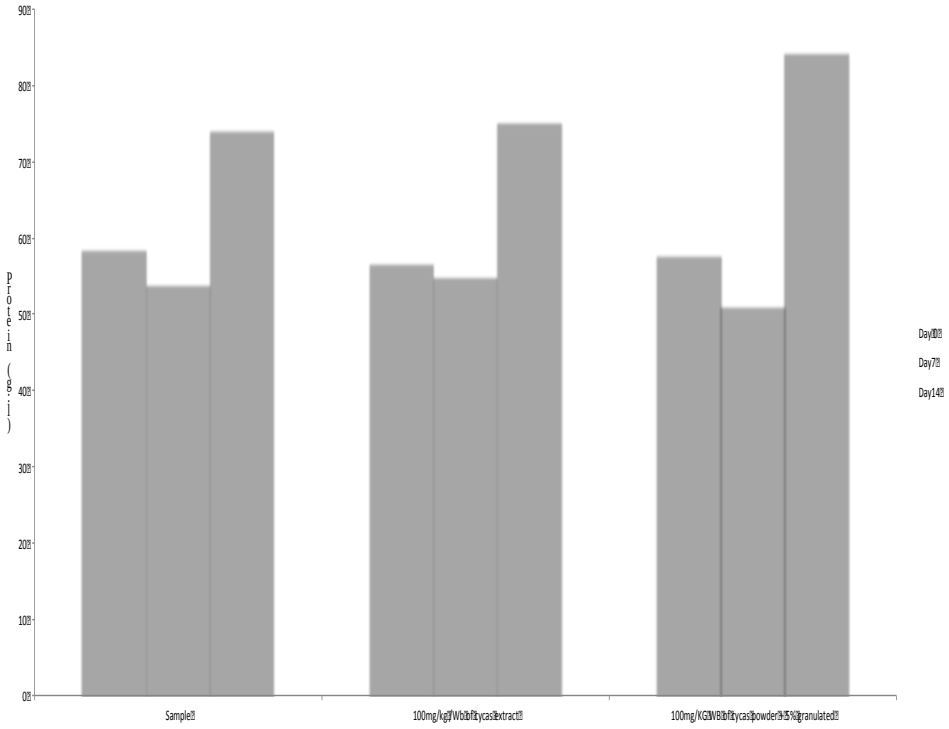

FIG. 5: Rate of total proteins in the blood as a function of time

Cycas does not affect the level of total proteins. This is clearly confirmed in the tissues of the cervix.In contrast, Cycas in general increased PAL activity significantly. However, it is noted that this increase is more pronounced in the case of the extract than that of the Cycas powder.

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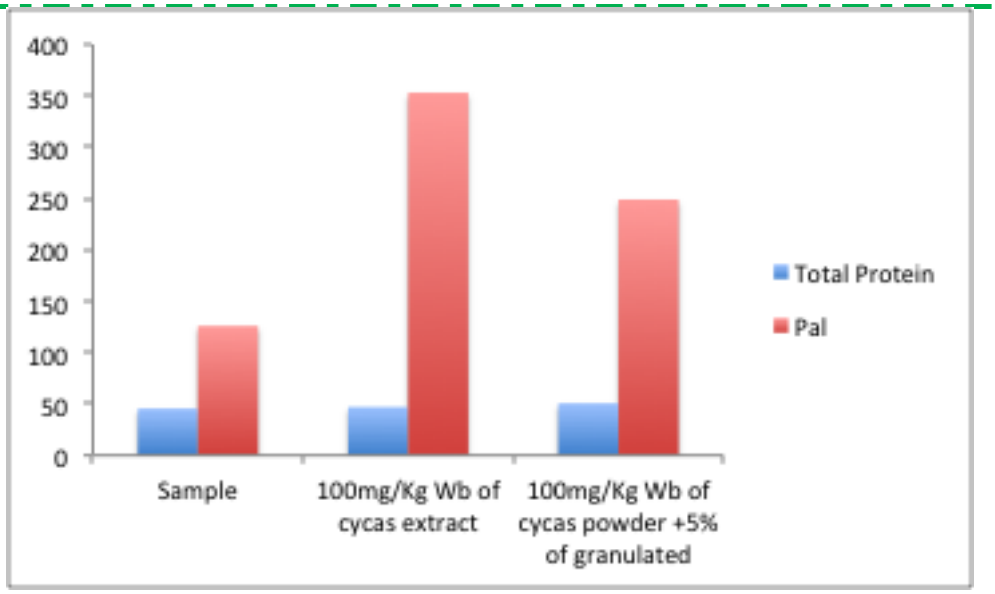

Figure 6: Changes in total protein and PAL on cervical tissue

The administration of the ethanolic extract of Cycas gives us a 50% result while with the Cycas powder we had 32% compared to the control which is 18%.The ethanolic extract of Cycas therefore increases the PAL / total protein ratio in the tissues of the cervix.

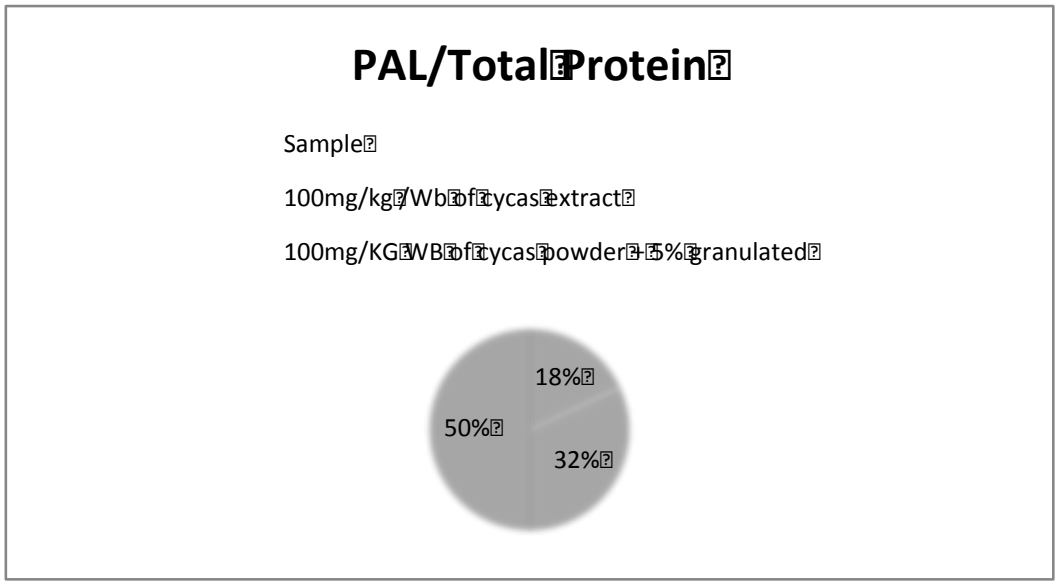

Figure 7: Variations in the PAL / Total Protein Ratio

Verification of the antiproliferative activity of the ethyl extract of the leaves of Hexalobus monopetalus.

Hexalobus monopetalus decreases the level of total proteins in the blood. This decrease depends on the time and is therefore a function of the exposure time.

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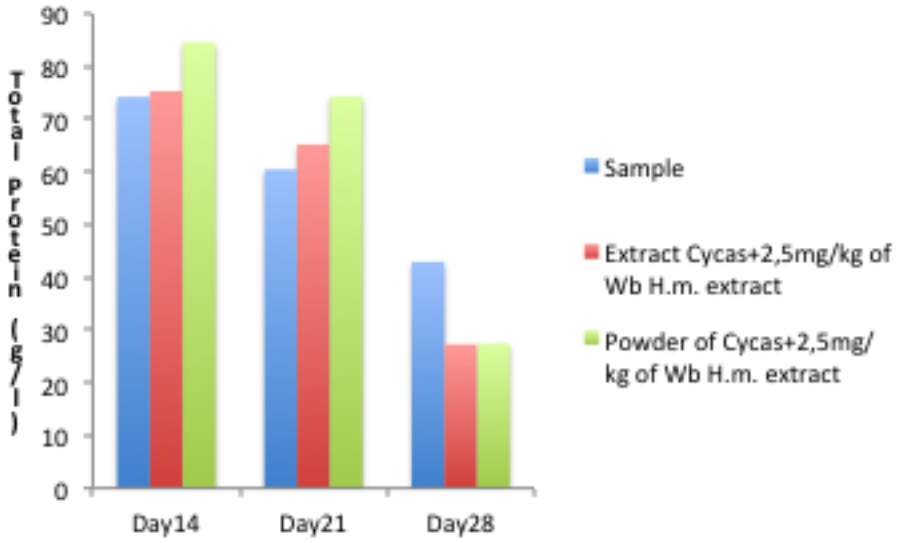

Figure 8: Changes in Total Protein Rate versus Time of Exposure with Hexalobus monopetalus

On the background of Cycas, it can be seen that Hexalobus monopetalus increases (tendency) the level of total proteins in the tissues of the cervix.Hexalobus monopetalus significantly reduced PAL levels in cervical tissue.This fall is more pronounced in the case of the Cycas powder than in that of the ethanolic extract.

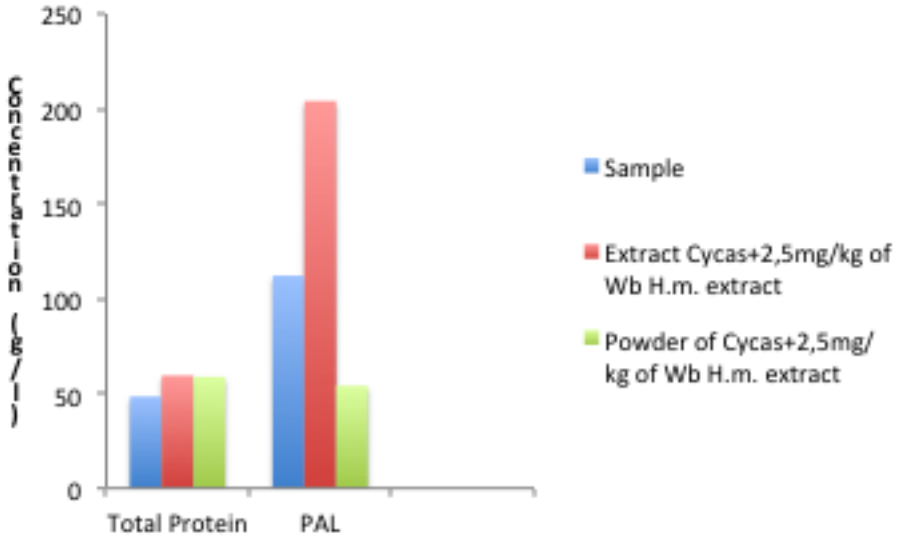

Figure 9: Variation of total protein and PAL on cervical tissue

Hexalobus monopetalus dropped the PAL / Total Protein ratio in the case of the use of Cycas powder.

As regards the ethanolic extract of Cycas, this ratio remains unchanged.

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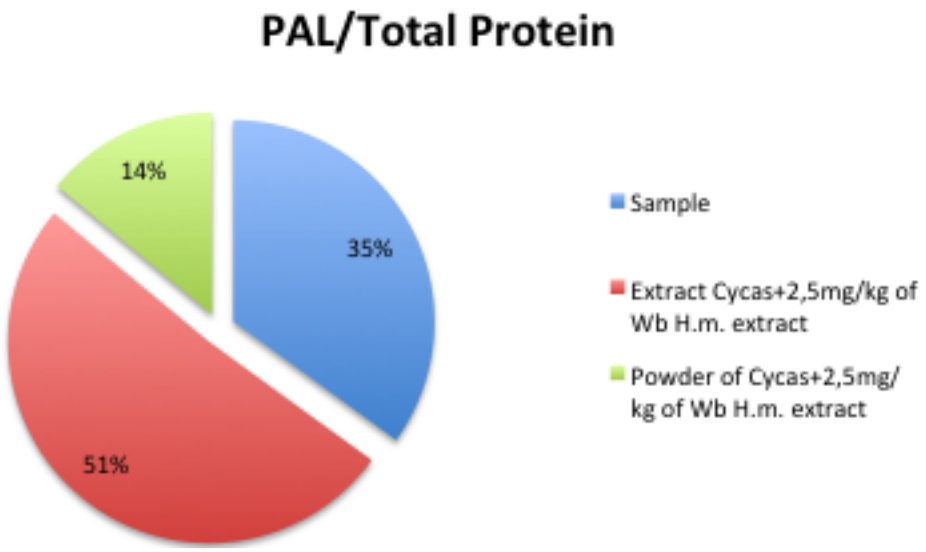

Figure 10: Change in PAL / total protein ratio in cervical tissue

Discussions

The cancerous disease is characterized by the progressive invasion of the organ of origin and then of the whole organism by cells that have become insensitive or insensitive to the mechanisms of tissue homeostasis and have acquired an indefinite proliferation capacity (immortalization) (RAPPILLARD A. 2010).

Cancer is an important cause of morbidity and mortality all over the world.

Cervical cancer, although declining in most developed countries in recent years, remains the second leading cause of mortality among women in developing countries (POINTREAU Y. et al., 2010) . Since the introduction of the cytological screening test and the establishment of screening programs for pre-cancerous lesions, the incidence and mortality due to cervical cancer have decreased significantly in industrialized countries. However, in most developing countries, the lack of a national plan for the prevention of this cancer, the lack of means and the lack of medical coverage of a large part of the population make this cancer a real problem of public health.

With cancer surgery and radiotherapy, cancer chemotherapy is now an essential part of the anti-cancer therapeutic arsenal of today (BRADY L.W. et al., 1993). In cervical cancer, the combination of chemotherapy and radiotherapy is the basic treatment (MAGNE N. et al., 2008). Despite advances in therapeutics and screening techniques, side effects of anti-tumor drugs, resistance problems and tumor escape remain the major causes of cancer treatment failure.

In recent years, pharmaceutical laboratories seeking new therapeutic molecules have invested heavily in the search for substances of natural origin (KINGHORN AD et al., 2009), to counteract the resistance of certain tumors to Anti-cancer agents on the one hand, and to develop anti-cancer therapies increasingly targeted to increase the specificity of chemo-therapeutic drugs. Since antiquity and thanks to their therapeutic properties, plants have played a crucial role in the art of healing. Today, they offer an interesting alternative in the field of drugs for the screening of potentially bioactive substances. In the field of anti-cancer agents, numerous cytotoxic molecules of plant origin are used in cancer chemotherapy (NEWMAN D.J and CRAGG G.M., 2007; NOBILI S. et al., 2009).

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The screening of new anticancer molecules is based in particular on the evaluation of their antiproliferative effects on cancerous cell lines in cultures (EISENBRAND G. et al., 2002). However, in vitro active compounds specific to certain types of tumors have a low potential toxicity and are believed to be active in vivo (BOYD M.R., 1989).

Hexalobus monopetalus (Annonaceae) commonly known as "Dankokwe (Fon)" is used in traditional medicine for various purposes. In diabetes, the aqueous decoctus of the roots is used in association with the trunks of Ficus glumosa orally (AKOUEGNINOU et al., 2006). Studies carried out in Benin (PIET A. LECLERCQ et al., 1960) on the essential oil obtained from the leaves of Hexalobus monopetalus (A. RICH) show that it is considered as a new source of citral, an organic compound possessing Calming virtues in the nervous system and muscles as well as sedative properties. On the other hand, Hexalobus monopetalus is an antiseptic and an antiviral. It would inhibit the promotion of skin cancers; However it would induce apoptosis.

On the basis of these data, we set ourselves the objective of evaluating the antiproliferative action of the leaves of Hexalobus monopetalus on cervical cancer in wistar rats.

Consequently, we carried out an ethanol extraction with the powder of the leaves of Hexalobus monopetalus and 90 ° ethanol. At the end of this extraction, we obtained a yield of 11.08%, which we consider to be low, compared to the yield obtained by AFFO B. and HOUEZE E. (2015) which is 33.78%. This low yield is due to the fact that the macera obtained after the filtration has not been reused by us in contrast to AFFO B. and HOUEZE E. (2015).

We also carried out an ethanol extraction with the powder of the leaves of Cycas revoluta and the ethanol 90 °. At the end of this extraction, we obtained a return of 10.2% which we consider average.

Cycas revoluta contains cyclosin (aglycone methyl azoxymethanol), which is known to induce liver cancer and many others (KAHN B.B., 1992). After ingesting a meal containing Cycas powder, intestinal bacteria hydrolyze the cycosine glucoside bond to release the aglycone, methyl azoxymethanol (WAMIDH H.T., 2011)

Since researchers have realized the effective carcinogenic properties of methyl azoxymethanol, these agents have been used to create reliable cancer models (to induce cancers) (LAQUEUR G.L., 1965). It is in this perspective that we have also used the leaf powder of Cycas revoluta, which is a plant of the same family as Cycas circinalis that is found in Benin, to induce cancer in our study.

In order to do this, we have firstly to verify the carcinogenicity of the powder and, on the other hand, the ethanolic extract of the leaves of Cycas revoluta. Thus we took 18 separate Wistars rats in 3 batches of 6 rats each. One served as controls and received only distilled water with a simple diet consisting of granules and tap water for 14 days; The second batch received a feed composed of granules and 100 mg / kg of the ethyl extract of Cycas revoluta; The third batch received a diet composed of 5% of Cycas revoluta powder and 10 mg / kg of cycas powder diluted in drinking water.

After the 14th day of gavage, two rats of each batch are sacrificed and dissected. The cervix was removed, crushed and centrifuged. The supernatant obtained after centrifugation was assayed for the biochemical parameters, Total Protein and Alkaline Phosphatase.

Note that the total proteins are assayed in the blood on days 0.7 and 14, respectively, before sacrificing the rats.Total proteins, which are the result of transcription of certain genes, their sudden increase, could translate abnormal and abnormal transcription and could characterize the carcinogenesis of the organ concerned (the cervix). On the other hand, alkaline phosphatases are enzymes; Substances capable of promoting a chemical reaction in our organism. They are present in the whole of our body and more particularly in organs like: the liver, the intestine, the kidneys, the uterus but also the bones and the white blood cells. The increase in alkaline phosphatases would result in the cell differentiation of the organ concerned (the cervix). The PAL product on total proteins shows that, compared with the

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control batch, the batch that received the cyclic ethyl extract grows much more than the batch that received the cycas powder.

We obtained, as a result of this experiment, that compared with the control rats which had received during the experimentation only distilled water accompanied by a simple diet comprising granules and tap water; A growth in total protein and alkaline phosphatase levels.

After this verification we looked for the antiproliferative activity of the ethyl extract of Hexalobus monopetalus leaves. For this we took the two remaining rats of the three batches to which we administered 2.5 mg / kg of the ethyl extract of the leaves of Hexalobus monopetalus diluted in 1 ml of 33% ethanol for 14 days.

The total proteins are dosed in the blood on days 0.7 and 14.

After the 14th day of gavage, each rat of each lot is sacrificed by sudden death and then dissected. The cervix is removed, crushed and centrifuged. The supernatant obtained after centrifugation undergoes the determination of the biochemical parameters, Total Protein and Alkaline Phosphatase.

There is an increase in the level of total proteins in the blood.

Conclusion

In the framework of the discovery of new therapeutic approaches to fight against cancer, we were interested in the search for natural substances derived from medicinal plants used in Beninese traditional medicine able to inhibit the proliferation of cancer cells of the cervix 'uterus.

Our studies have shown that the administration of cyclic revoluta leaves for 14 days of 10 mg / kg body weight may induce proliferation of cancer cells in the cervix; also 100mg / kg body weight of the powder of Cycas revoluta would induce the proliferation of cancer cells of the cervix. In both species cases, we noted increased proliferation of cancer cells in the cervix, resulting in low total protein and high alkaline phosphatase.

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