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Assessment of Programmed Cell Death of *Aspergillus flavus* by Triggered Cysteine-dependent Aspartate-directed Proteases (Meta-caspase3) Lethality Mechanism of Novel Compounds Isolated from Ethyl Acetate Extract of *Spondias mombin*

Oludare Temitope Osuntokun[1], Olaposi I Omotuyi[2], Anthonia O Oluduro[3], Thomas O Idowu[4]

Affiliation

[1]Department of Microbiology, Faculty of Science, Adekunle Ajasin University, Akungba Akoko, P.M.B 001, Ondo State, Nigeria
[2]Centre for Biocomputing and Drug Development (CBDD), Adekunle Ajasin University, Nigeria
[3]Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Osun, Nigeria
[4]Department of Pharmaceutical Chemistry, Obafemi Awolowo University, Ile-Ife, Osun, Nigeria

***Corresponding author:** Oludare Temitope Osuntokun, Department of Microbiology, Faculty of Science, Adekunle Ajasin University, Akungba Akoko, P.M.B 001, Ondo State, Nigeria, E-mail: osuntokun4m@yahoo.com

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Abstract

Spondias mombin is a plant that has been traditionally noted for its medicinal with a preliminary results report a wide range of antibacterial and antifungal properties. Meta-caspases and Caspases are essential in cells for programmed cell death, in development and most other stages of adult life, and have been termed "executioner" proteins for their roles in the cell. A 12 hours old culture of each microorganism was re-suspended in plant extract at 1000 µg mL in a total volume of 500 µl for 0, 15, 30, 45, 60, and 180 minutes. The cells were pelleted by centrifugation at 5000 g for 5 minutes. The pellets were rinsed twice in phosphate buffer saline (PBS). Then 1/10 volume of 95% ethanol plus 5% saturated phenol were added to the pellets to stabilize cellular RNA. The cells were then re-harvested by centrifugation (8200 g, 4°C and 2 minutes). The supernatant was aspirated and pellets re-suspended in 800 µl of lysis buffer (10 mMTris, adjusted to pH 8.0 with HCl, 1 mM EDTA) and 8.3 U/ml Ready-LyseTM Lysozyme Solution. After the pellets were re-suspended, 80 µl of a 10% SDS solution was added, mixed and incubated for 2 minutes at 64 °C. Then 88 µl of 1 M NaOAc (pH 5.2) was mixed with the lysate followed by an equal volume of water and saturated phenol was added. Total RNA was quantified using Spectrophotometric absorbance at 260 nm DNA was removed with Turbo DNA-free (Ambion, Inc.). Reverse Transcription-PCR reaction was performed in a 15.0 µl final volume (kit number-DNA-PCR739288). Assessment of Polymerase Chain Reaction products (amplicons) were electrophoreses in 0.5% of agarose gel using 0.5 × TBE buffer (2.6 g of Tris base, 5 g of Tris boric acid and 2 ml of 0.5M EDTA and adjusted to pH 8.3 with the sodium hydroxide pellet) with 0.5 µl ethidum bromide. The mechanism of action of isolated novel compounds using Metacaspase3 to programme the death of test organism (*Aspergillus flavus*) between 0 and 180 minutes interval. It was observed that cell (via DNA) were completely destroyed at 180 minutes with all the isolated compounds. The purpose of this research work is to evaluate the programmed cell death (PCD) of *Aspergillus flavus* by triggered Cysteine-dependent Aspartate-directed proteases (meta-caspase3) lethality mechanism of novel compound isolated from ethyl acetate extract of *Spondias mombin*.

Keywords: Programmed Cell Death, *Aspergillus flavus*, Cysteine-dependent Aspartate-directed proteases (Meta-caspase3), *Spondias mombin*, Novel compound (A1- Epigallocatechin, Epicatechin and Stigmasterol phytosterol (Synergy),A3-Aspidofractinine-3-methanol and F3-Terephthalic dodecyl 2-ethylhexyl ester).

Introduction

Spondias mombin is a plant that has been traditionally noted for its medicinal and food values. Preliminary results report a wide range of antibacterial and antifungal properties [1, 2]. Scientific investigations have shown that it has anthelmintic, antioxidant, antimicrobial and anti- inflammatory actions [3, 4]. Meta-caspases can be defined as cysteine-dependent aspartate-directed proteases are a family of cysteine proteases that play essential roles in apoptosis (programmed cell death), necrosis, and inflammation. Caspases are essential in cells for apoptosis, or programmed cell death, in development and most other stages of adult life, and have been termed "executioner" proteins for their roles in the cell. Some caspases are also required in the immune system for the maturation of lymphocytes. Failure of apoptosis is one of the main contributions to tumor development and autoimmune diseases; this, coupled with the unwanted apoptosis that occurs with ischemia or Alzheimer's disease, has stimulated interest in caspases as potential therapeutic targets. Inactive protease of caspase family is in state of pro-enzyme which amino acid end has a sequence called “pro-

domain". When zymogen is being activated, the pro-domain is cleaved and the rest part is cut into two subunits called P20 and P10. Active zymogens consist of these two subunits in forms of (P20/P10)2. This activation reaction is also Asp-specific for reason that the cleavage occurs between Asp of conserved sequences in pro-enzyme and the amino acids sequence after Asp. In the cleavage the small subunit at the carboxyl end is cleaved first and pro-domain is then cut off the amino side of the big subunit. The cleavage can be self-catalyzing of pro-enzyme and mediated enzyme or functioned by other proteases of ICE family.

Pro-caspase-3 has 277 amino acids, molecular weight of 32kD, 30% homology with ICE and 35% homology with CED-3. In caspase family pro-caspase-3 is the most homologous to CED-3 both in structure and substrate specificity. The pro-domain of caspase-3 is shorter than that of ICE which has 28 amino acids, but its activity center and conserved amino acids that are related with substrate binding are the same with ICE. In activation, pro-caspase-3 is cleaved at two sites: Asp28~Ser29 and Asp175~Ser176, giving rise to two fragments: P17 (29~175) and P10 (182~277) which are close to P20 and P10 of ICE. The two subunits combine and form active caspase-3. When being activated, pro-caspase-3 is not active of catalyzing before being cleaved by granzyme B (GrzB) or caspase-10 at D175. Other caspases e.g. ICE might participate in cleaving pro-domain of caspase-32.0 [4].

Caspase-3 is indispensible in apoptosis. It triggers apoptosis when being transfected into insect Sf9 cells. This process can be blocked by BCL-2. Exclusion of caspase-3 in extractions of apoptotic cells leads to loss of capability of inducing apoptosis. Adding of caspase-3 let it regain the capability of inducing apoptosis. Caspase-3 can be activated by various factors. In CTL-mediated killing, caspase-3 can be activated both by Fas/FasL pathway and by granzyme B pathway. Granzyme B is a kind of serine esterase in cells, and is the only protease that cleaves after Asp except caspases in mammals. Granzyme B can specifically cleave IxxD sequence at the C terminal of catalyzing subunit of ICE family and activate caspase-2, 3, 6, 7, 8, 9, 10. ICE can be cleaved by granzyme B, too, but it can't be activated after cleavage. The purpose of this research work is to evaluate the Programmed Cell Death (PCD) of *Aspergillus flavus* by triggered Cysteine-dependent Aspartate-directed proteases (meta-caspase3) lethality mechanism of novel compound isolated from ethyl acetate extract of *Spondias mombin*.

Materials and Method

Microorganism for the research work

The strains used for this research work were fungi collected from Central Medical Laboratory (CML), Obafemi Awolowo University Teaching, Hospital (OAUTH), Ile Ife, Osun State, and the Institute of Advance Medical Research and Training (IMRAT), University College Hospital, Ibadan, Oyo State Nigeria.

Sources of microorganisms

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Authentication of test microorganisms

The identity of the test organisms was confirmed ID 32 C system (Biomerieux, France) following the manufacturer's instructions. The yeast isolates were identified by the ID 32 C Analytical Profile Index [6].

Isolation of RNA

A 12 hours old culture of each microorganism was re-suspended in plant extract at 1000 µg mL in a total volume of 500 µl for 0, 15, 30, 45, 60, and 180 minutes. The cells were pelleted by centrifugation at 5000 g for 5 minutes. The pellets were rinsed twice in phosphate buffer saline (PBS). Then 1/10 volume of 95% ethanol plus 5% saturated phenol were added to the pellets to stabilise cellular RNA. The cells were then re-harvested by centrifugation (8200 g, 4 °C and 2 minutes). The supernatant was aspirated and pellets re-suspended in 800 µl of lysis buffer (10 mMTris, adjusted to pH 8.0 with HCl, 1 mM EDTA) and 8.3 U/ml Ready-LyseTM Lysozyme Solution. After the pellets were re-suspended, 80 µl of a 10% SDS solution was added, mixed and incubated for 2 minutes at 64 °C. Then 88 µl of 1 M NaOAc (pH 5.2) was mixed with the lysate followed by an equal volume of water and saturated phenol was added. This was incubated at 64°C for 6 minutes while inverting the tubes every 40 seconds. The aqueous phase was separated following centrifugation at 21, 000 g for 10 minutes at 4°C.
The RNA was precipitated from the aqueous layer using 1/10 volume of 3 M NaOAc (pH 5.2), 1 mM EDTA and 2 volumes cold EtOH and centrifugation at 21, 000 g for 25 minutes at 4°C. Pellets were washed with ice cold 80% EtOH and centrifuged at 21, 000 g for 5 minutes at 4°C. The ethanol was carefully removed and the pellets were air dried for 20 minutes. The pellets from each split sample were re-suspended in a total of 100 µl of RNase-free water and combined into one microfuge [7] (kit number-DNA-PCR739288).

Synthesis of convertible (cDNA)

Total RNA was quantified using spectrophotometric absorbance at 260 nm DNA was removed with Turbo DNA-free (Ambion, Inc.). Removal of DNA from the RNA samples was performed using DNA-free™ DNA Removal Kit (ThermoFisher) following manufacturer's protocol. Purified DNA-free RNA was converted to cDNA immediately using ProtoScript® First Strand cDNA Synthesis Kit (NEB). The cDNA was diluted to a final volume of 286 µl and stored at 4°C (8).

PCR protocol

Reverse Transcription-PCR reaction was performed in a 15.0 µl final volume. Briefly, 1 µl template cDNA (~40 ng) was combined with 1.0 µl of forward primer (5 nM), 1.0 µl of reverse primer (5 nM), 4.5 ml nuclease-free water and 7.5 µl of Taq 2X Master Mix. Thermo cycling was performed by 40 cycles at 95 °C for 15 seconds, 60 °C for 15 seconds and 72 °C for 15 seconds. Analysis of the PCR products was performed using 1.5% agarose gel solution in TBE buffer and visualisation was enabled by soaking gel in ethidium bromide solution for 10 minutes and UV-transilluminator. The data obtained were analyed using Graph pad prism version 6.01 description and frequency. statistic was generated to describe the diameter of inhibition. quantitative phytochemical constituent and toxicological prameter to test for the level of significance [8].

Gel electrophoresis

Assessment of Polymerase Chain Reaction products (amplicons) were electrophoresed in 0.5% of agarose gel using 0.5 × TBE buffer (2.6 g of Tris base, 5 g of Tris boric acid and 2 ml of 0.5M EDTA and adjusted to pH 8.3 with the sodium hydroxide pellet) with 0.5 µlethidum bromide. The expression product was visualized as bands by UV-transilluminator [8-10] (**Table 1 & Chart 1**).

Target genes or Biomarkers	Meta-caspase3
FORWARD 5'-3'	AAAGACG CTAAGCCCAACGA
REVERSE 5'-3'	ATCCGGTGCATCTCATCGTC
References	(10)

Table 1: Primers used for PCR molecular investigation, to determine the mechanisms of action of novel compound extracted *from Spondias mombin* on *Aspergillus flavus.*

Test Organisms ***Aspergillus flavus*** using a modified hot SDS/hot phenol method (12 hours old culture)

↓

Microorganism was re-suspended in plant extract at 1000 µg mL in a total volume of 500µl for 0, 15, 30, 45, 60, and 180 minutes

↓

The cells were pelleted by centrifugation at 5000g for 5 mins, Pellets were rinsed twice in phosphate buffer saline (PBS)

↓

1/10 volume of 95% ethanol plus 5% saturated phenol were added to the pellets to stabilise cellular RNA.

↓

The cells were then re-harvested by centrifugation (8200 g, 4°C and 2 minutes).

↓

The supernatant was aspirated and pellets resuspended in 800 µl of lysis buffer (10 mMTris, adjusted to pH 8.0 with HCl,1 mM EDTA) and 8.3 U/ml Ready-LyseTM Lysozyme Solution.

↓

Pellets were resuspended, 80 µl of a 10% SDS solution was added, mixed and incubated for 2 minutes at 64°C

↓

88 µl of 1 M NaOAc (pH 5.2) was mixed with the lysate followed by an equal volume of water and saturated phenl was added.

↓

Incubated at 64°C for 6 minutes while inverting the tubes every 40 seconds

↓

aqueous phase was separated following centrifugation at 21,000 g for 10 minutes at 4°C.

↓

RNA was precipitated from the aqueous layer using 1/10 volume of 3 M NaOAc (pH 5.2), 1 mM EDTA and 2 volumes cold EtOH and centrifugation at 21,000 g for 25 minutes at 4°C.

↓

Pellets were washed with ice cold 80% EtOH and centrifuged at 21,000 g for 5 minutes at 4°C.

↓

Ethanol was carefully removed and the pellets were air dried for 20 minutes. The pellets from each split sample were re-suspended in a total of 100 µl of RNase-free water and combined into one microfuge

Chart 1: Isolation of RNA from bacterial cell (Osuntokun et al., 2017).

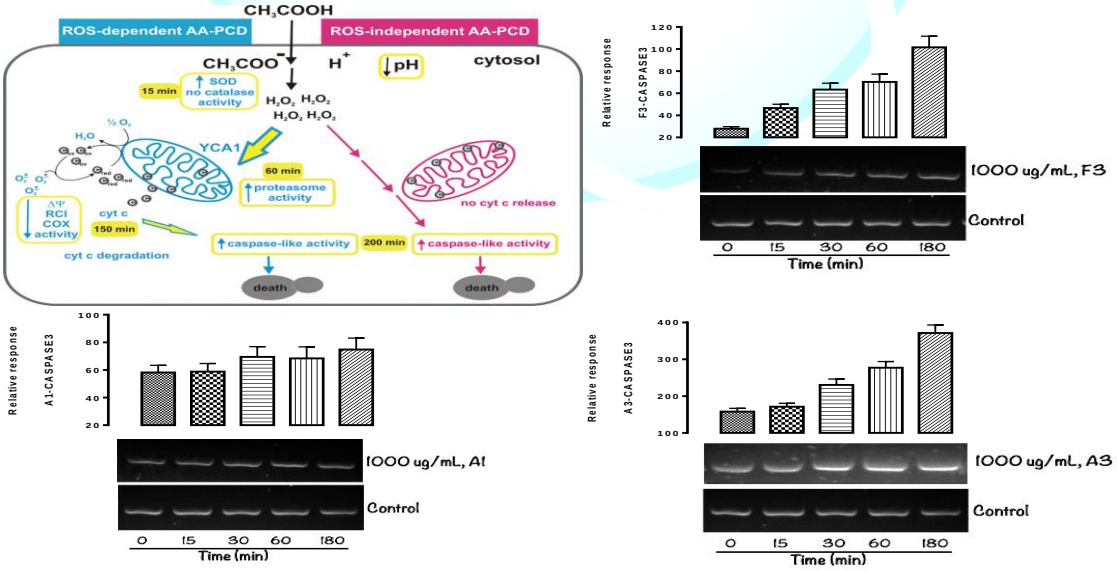

Figure 1: Agarose gel electrophoresis of the amplification of Product Coding Metacaspase3 (2861bp) selected interaction between gene and *Aspergillus flavus* (Ldder DN 100bp) (A-C) and isolated novel compounds from ethyl acetate extract of *Spondias mombin*.

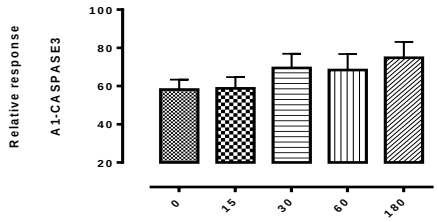

Figure 1a: Mechanism of Action of Isolated novel Compound A[1]-(Epigallocatechin, Epicatechin and Stigmasterol Phytosterol (Synergy) from ethyl acetate extract of *Spondias mombin* using Gene Expression Metacaspase3 Lethality, to Program the Cell Death (PCD) of *Aspergillus flavus*.

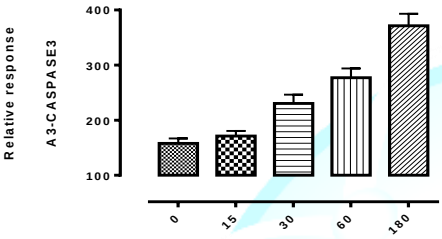

Figure 1b: Mechanism of Action of Isolated novel Compound A[3]-(Aspidofractinine-3-methanol) from ethyl acetate extract of *Spondias mombin* using Gene Expression Meta caspase3 Lethality, to Program the Cell Death (PCD) of *Aspergillus flavus*.

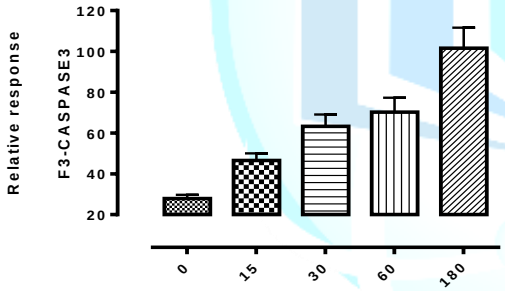

Figure 1c: Mechanism of Action of Isolated novel Compound F[3]-(Terephthalic dodecyl 2-ethylhexyl ester) ethyl acetate extract of *Spondias mombin* using Gene Expression Metacaspase3 Lethality, to Program the Cell Death (PCD) of *Aspergillus flavus.*

Results

Programmed Cell Death of *Aspergillus flavus* Trigger Meta-caspase3 Lethality mechanism. **Figure 1** shows the mechanism of action of isolated novel compounds using Metacaspase3 to programme the death of test organism (*Aspergillus flavus*) between 0 and 180 minutes interval. It was observed that cell (via DNA) were completely destroyed at 180 minutes with all the isolated compounds from *Spondias mombin*. The graphically represented in **Figures 1, 1a, 1b,** and **1c** of isolated novel compounds and Programmed Cell Death in *Aspergillus flavus* trigger meta-caspase3 Lethality by *Aspergillus flavus* were demonstrated below. It can be deduced that the isolated novel compounds has fungicidal mechanism of action using triggered Meta-caspase3 lethality to program the death of the test fungus (*Aspergillus flavus*).

Discussion

The purpose of this research work is to evaluate the Programmed Cell Death (PCD) of *Aspergillus flavus* by triggered Cysteine-dependent Aspartate-directed proteases (meta-caspase3) lethality mechanism of novel compound isolated from ethyl acetate extract of *Spondias mombin*. The isolated compound are A1- Epigallocatechin, Epicatechin and Stigmasterol phytosterol (Synergy), A3-Aspidofractinine-3-methanol and F3-Terephthalic dodecyl 2-ethylhexyl ester. Apoptosis or Programmed Cell Death (PCD) is a prominent feature of a developing cell signalling. Metacaspase3 is a caspase protein that interacts with caspase-8 and caspase-9. It is a protein and member of cysteine-aspartic acid protease. Sequential activation of caspase plays a central role in the execution phase of cell apoptosis [11]. In this present study, the mechanism of Caspase3 was discussed as mention by previous research [12].

It was reported that Caspase involved the catalytic site and sulfohydyl group of cys-285 and the imidazole ring of His 237-His 237 stabilizes the carbonyl group of the key aspartate residue while cys 285 attacks to ultimately cleaves the peptide bond. Cys 285 and Gily 238 also function to stabilize the tetrahedral transition state of the substrate enzyme complex through hydrogen bonding [13].

In figure 1, 1a, 1b and 1c the breakdown of the pathway mechanism are as follow. Amino Acid (AA) enters *Aspergillus flavus* cells by diffusion through the plasma membrane. In the cytosol, AA dissociates into Acetate and protons causing intracellular acidification. Alternative PCD pathways are induced by Amino acid (AA): a ROS-dependent (blue lines) and a ROS-independent (pink lines) pathway. H2O2 accumulates early in both the pathways. In the ROS-dependent pathway SOD activity increases at 15 min. YCA1 acts upstream of cyt c (c) release from mitochondria to the cytosol; released cyt c acts as an electron donor (cred) to mitochondrial respiratory chain and as superoxide anion (O2•−) scavenger (cox) and is degraded by unidentified proteases in a late phase; mitochondrial functions progressively decline as judged by decrease in mitochondrial membrane potential, Respiratory Control Index (RCI) and COX activity; caspase-like activity increases in a late phase with a complete loss of cell viability at 200 min. In the ROS-independent AA-PCD pathway, cyt c is not released into the cytosol but the caspase-like activity increases in a late phase (14).

In figure 1, Metacaspase3 is activated in the apoptotic cell both by extrinsic (Death ligand) and intrinsic (mitochondrial pathway) [15]. The mitochondrial pathway was demonstrated in the fig 1. In mitochondrial pathway, Zymogen features of Caspase3 is necessary, if unregulated Caspase actively would kill cell indiscriminately. As an executioner Caspase, the Caspase-3 zymogen has virtually no activity until it is cleared by an initiator Caspase after apoptotic signalling events like the inclusion of isolated compound A1, A3 and F3. On inclusion, it can activate initiator Caspases into cells targeted for apoptosis by killer T cells [14, 15] In Metacaspase3, A1, A2 and F3, it was observed that there is a complete inhibition of *Aspergillus flavus* (fungi) and their death phase were adequately measured between 0 and 180 minutes interval.

The *Aspergillus flavus* relative response was demonstrated by the graph in the figure 1, 1a, 1b and 1c, at 0-180 mins interval.it was observed that major substrate of metacaspase-3 is Poly ADP-ribose polymerase (PARP) which was found in *Aspergillus flavus*. It correlates with DNA repairmen and monitoring of gene integration. It was reported by

Boutright and Salvesen (2003) that metacaspase 3 exist as inactive pro-enzymes that undergo proteolytic processing at conserved aspartic residues to produce two subunits large and small that dimerize to form the active enzyme, this protein cleaves and activates caspase 6 and 7 and two protein itself is processed and activated by metacaspases 8, 9 and 10. It is the predominant metacaspase involved in the cleavage of amyloid beta for a precursor protein which is associated with neuronal death. Alternative splicing of this gene results in the two transcript variants that encode the same protein [16].

The mechanism of apoptosis is highly complex and involves energy dependent cascade of molecular events. It is mediated mainly through three pathways: extrinsic, intrinsic and perforin pathway. The apoptotic mode of cell death is an active and defined process which plays an important role in the development of multicellular organisms and in the regulation and maintenance of the cell populations in tissues upon physiological and pathological conditions. During the initiation of apoptosis in this study, PARP 116kD is cleaved by metacaspase3 into two fragments, 31kD and 85kD at Asp216-Gly217, separating its two zinc finger domain that binds with DNA from its catalyzing domain of carboxyl end, and loses its normal function. Then the activity of endonuclease which is down regulated by PARP and dependent on Ca_{2+}/Mg_2 increases, and DNA in nucleosomes is lysed which triggers apoptosis which can be observed during the course of the research. This lysis process can be inhibited by Ac-DEVD-CHO, a specific inhibitor of metacaspase-3, but can't be inhibited by CrmA. Metacaspase-3 can also cleave U1-70K、DNA-PK、PKCd and PKCq. Both PKCd and PKCq belong to novel PKC (nPKC). After being cleaved by metacaspase-3 which cuts off the regulation domain, they become active PKC. Moreover, over expression of PKCd and PKCq can trigger apoptosis, which illustrates they participate in inducing of apoptosis [17].

Sokolov et al reported in vitro metacaspase 3 was found to prefer the peptide sequence DEXDG (Asp-Glu-Val-Asp-Gly) with cleavage occurring on the carboxy side of the second aspartic acid residue (between D and G) metacaspase 3 is active over a broad pH range that is slightly higher (more basic) than many of the other executioner metacaspases, this broad range indicates that mtacaspase3 will be fully active under normal and apoptotic cell condition .It is important to discuss the activation of this programmed cell death, this has helped to measure the inhibitor factors of isolated compound A1, A3 and F3 on selected microbe during this research work [18,19].

In Extrintics activation, it will trigger the hallmark Caspase-cascade characteristic of the apoptotic pathway, in which metacaspase 3 plays a dominant role. It should be noted in the scope of this research work and reported by previous authors that mitochondria works in combination with caspase 9, apoptosis-activating factors 1 (Apat 1) and ATP to process procaspase 3, these molecules are sufficient to activate caspase 3 invitro but other regulator proteins are necessary in vivo [20]. The major substrate of metacaspase 3 is poly ADP ribose polymerase (PARP), it correlates with DNA repairmen, damages and monitoring of gene integration to initiate, all this were clearly stated in the Figures 1, 1a, 1b and 1c.

In conclusion, Cysteine-dependent Aspartate-directed proteases (meta-caspase3) lethality is the best method to measure the mechanism of action of lethal compound from *spondias mombin* isolated compound on microbe cells and to demonstrate lethality and mechanism of the isolated compound on the cell of *Aspergillus flavus*. This is an Alternative to after the early burst of intracellular H_2O_2 accumulation, AA-PCD can proceed via a ROS- and YCA1-independent pathway, in which the death rate is faster than that of the ROS-dependent pathway, cytc is not released, but still a late caspase-like activity increase is observed which is not affected by H_2O_2 scavengers, such as N-acetyl-L-cysteine, therefore *Aspergillus flavus* can used to demonstrate effects of Cysteine-dependent Aspartate-directed proteases (meta-caspase3) lethality.

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