Bacteriostatic and Bactericidal Mechanism of Novel Compound Isolated from Ethyl Acetate Stem Bark Extract of *Spondias mombin* Using Biomarker Repressor LexA gene on *Escherichia coli* and *Bacillus subtilis*

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## Abstract

The purpose of the research work is to determine the mechanism of action of a novel compound (A1-Epigallocatechin, Epicatechin and Stigmasterol Phytosterol (Synergy)), (A3-Aspidofractinine-3-methanol) and (F3-Terephthalic acid, dodecyl 2-ethylhexyl ester) against two selected clinical organism by Repressor LexA gene expression. Clinical microorganisms were cultured and sub-culturing in Department of Microbiology and Centre for Biocomputing and Drug Development (CBDD), Adekunle Ajasin University, Akungba Akoko, Ondo-state, a 12 hours old culture of each microorganism was re-suspended in *Spondias mombin* extract at 1000 µg mL in a total volume of 500 µl for 0, 15, 30, 45, 60, and 180 minutes. The bacteria cells were pelleted by centrifugation at 5000 g for 5 minutes, to isolate their DNA. Total RNA was quantified using spectrophotometric absorbance at 260 nm. DNA was removed with Turbo DNA-free (Ambion, Inc.) and removal of DNA from the RNA samples was performed using DNA-free™ DNA Removal Kit. Reverse transcription–PCR reaction was performed in a 15.0 µl final volume 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. The master mix and the aliquot were assessed by Polymerase Chain Reaction products (amplicons) and 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 0.5 M EDTA and adjusted to pH 8.3 with the sodium hydroxide pellet) with 0.5 µl ethidium bromide. It was observed that, in 30 minutes time interval, the mechanism of action is bacteriostatic on *B. subtilis*, an SOS regulum was activated by *B. subtilis* at 180 minutes, to resuscitate the dying cell of *B. subtilis*. The mechanism of action of biomarker repressor LexA gene on isolated novel compounds using *E. coli* as the test bacteria. It was observed that isolated novel compounds has bactericidal action on *E. coli* at 180 minutes of administration using repressor LexA gene to stimulate death phases of growth. The novel compounds disrupt the SOS regulon, the *E. coli* cells were complete destroyed. *LexA* has rapid progress to sequence death phase with time interval, this led to the death of the *E. coli* cell, it can be deduced that, this method can be used to program the death of bacteria cell in case of a recalcitrant resistance infections in the treatment of severe cough, diarrhea, dysentery, haemorrhoids, gonorrhea and leucorrhoea.

**Keywords:** Epigallocatechin; Epicatechin; Stigmasterol phytosterol; Repressor LexA

## Introduction

*Spondias mombin* Linn is a small tree that grows up to 30 m high and 1.5 m in height, moderately buttressed; bark thick, corky, deeply fissured, slash pale pink, darkening rapidly, branches low, branchlets glabrous; leaves pinnate, leaflets 5-8 opposite pairs with a terminal leaflet. It belongs to the family Anacardiaceae and the fruits have a sharp, acid taste [1]. The roots are also used as febrifuge and stem bark is used as a purgative and in local applications for leprosy. The stem bark decoction is also used in the treatment of severe cough and used for diarrhea, dysentery, haemorrhoids and a treatment for gonorrhoea and leucorrhoea [2]. The decoction of the astringent bark is believed to expel calcifications from the bladder [3].

A report showed that the stem bark contains a certain amount of tannin, alkaloid and flavonoid, this explains the reason why the dry pulverized stem bark is applied as a dressing to a wound [4]. The stem bark is a remedy for diarrhea and dysentery. The gum is employed as an expectorant and to expel tapeworms [5,6]. A leaf infusion is a common cough remedy or used as a laxative for fever with constipation and the pounded leaves of *S. mombin* is used as an eye lotion and the juice pressed from young, warm leaves is given to children for stomach troubles [7]. A tea made from the flowers and leaves is taken to relieve stomach ache, biliousness, urethritis, cystitis and eye and throat inflammations. A decoction of the root is used as purgative [8,9].

Repressor LexA is a transcriptional repressor SOS response genes coding primarily for error-prone DNA polymerases, DNA repair enzymes and cell division inhibitors [10]. *LexA* forms de facto a two-component regulatory system with *RecA*, which senses DNA damage at stalled replication forks, forming monofilaments and acquiring an active conformation capable of binding to *LexA* and causing *LexA* to cleave itself, in a process called autopoiesis [11]. DNA damage can be inflicted by the action of antibiotics plant extract like *Spondias mombin* [12] of potential clinical interest is the induction of the SOS response by antibiotics. Bacteria require topoisomerases such as DNA gyrase or topoisomerase IV for DNA replication.
Antibiotics and plant extract like *Spondias mombin* are able to prevent the action of these molecules by attaching themselves to the gyrase-DNA complex, leading to replication fork stall and the induction of the SOS response. The expression of error-prone polymerases under the SOS response increases the basal mutation rate of bacteria. While mutations are often lethal to the cell, they can also enhance survival. In the specific case of topoisomerases, some bacteria have mutated one of their amino acids so that the plant extract can only create a weak bond to the topoisomerase. This is one of the methods that bacteria use to become resistant to plant extract. Plant extract treatment can therefore potentially lead to the generation of mutations that may render bacteria resistant to plant extract.

In addition, plant extract has also been shown to induce *via* the SOS response dissemination of virulence factors [13] and antibiotic resistance determinants, as well as the activation of integron integrases [14] potentially increasing the likelihood of acquisition and dissemination of antibiotic resistance by bacteria. *LexA* biomarker contains a DNA binding domain. The winged HTH motif of *LexA* is a variant form of the helix-turn-helix DNA binding motif it is usually located at the N-terminus of the protein [15]. The mode of action of Repressers *dinA*, *dinB*, *dinC*, *recA* genes and itself by binding to the 14 bp palindromic sequence 5′-CGAACNNNNGTTCG-3′; some genes have a tandem consensus sequence and their binding is cooperative. In the presence of single-stranded DNA, *RecA* causes an autocatalytic cleavage which disrupts the DNA-binding part of *LexA*, leading to derepression of the SOS regulon and eventually DNA repair; autocleavage is maximal at pH 11 in the absence of *RecA* and ssDNA [16].

**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 (PBE). Then 1/10 volume of 95% ethanol plus 5% saturated phenol (PBE). Then 1/10 volume of 95% ethanol plus 5% saturated phenol (PBE). Then 1/10 volume of 95% ethanol plus 5% saturatedphenol were added to the pellets to stabilise cellular RNA. The supernatant was aspirated and pellets re-suspended in 800 µl of lysis buffer (10 mM Tris, adjusted to pH 8.0 with HCl, 1 mM EDTA) and 8.3 U/ml Ready-Lyse™ 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 [18] (Table 1).

**Synthesis of convertible**

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 Protocol™ First Strand cDNA Synthesis Kit (NEB). The cDNA was diluted to a final volume of 286 µl and stored at 4°C [19].

**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 µl 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 (Table 2). 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 analyzed using Graph pad prism version 6.01 descriptions and frequency. Statistics were generated to describe the diameter of inhibition, quantitative phytochemical constituent and toxicological parameter to test for the level of significance [20].

**Materials and Methods**

**Isolation of clinical microorganisms**

Clinical microorganisms were isolated and cultured from Department of Microbiology and Centre for Biocombing and Drug Development (CBBD), Adekunle Ajasin University, Akungba Akoko, Ondo-state, Nigeria. High quality RNA was isolated from the selected test organisms (*E. coli*, *B. substils*, *A. flavus*) using a modified hot SDS/hot phenol method [17].

**Isolation of RNA**

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Steps | Procedure
--- | ---
1 | Test Organisms (E. coli, B. subtilis) using a modified hot SDS/hot phenol method (12 hours old culture).
2 | 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.
2 | The cells were pelleted by centrifugation at 5000 g for 5 minutes, Pellets were rinsed twice in phosphate buffer saline (PBS).
4 | 1/10 volume of 95% ethanol plus 5% saturated phenol were added to the pellets to stabilise cellular RNA.
5 | The cells were then re-harvested by centrifugation (8200 g, 4°C and 2 minutes).
6 | The supernatant was aspirated and pellets resuspended in 800 µl of lysis buffer (10 mM Tris, adjusted to pH 8.0 with HCl, 1 mM EDTA) and 8.3 U/ml Ready-LyseTM Lysozyme Solution.
7 | Pellets were resuspended, 80 µl of a 10% SDS solution was added, mixed and incubated for 2 minutes at 64°C.
8 | 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.
9 | Incubated at 64°C for 6 minutes while inverting the tubes every 40 seconds.
10 | Aqueous phase was separated following centrifugation at 21,000 g for 10 minutes at 4°C.
11 | 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.
12 | Pellets were washed with ice cold 80% EtOH and centrifuged at 21,000 g for 5 minutes at 4°C.
13 | 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.

Table 1: Steps for isolation of RNA from bacterial cell.

Figure 1: Agarose gel electrophoresis of the amplification of product coding LexA (620 bp) on selected interaction between gene and cells (Ldder DN 100 bp). Mechanism of action of isolated compounds A1, A3 and F3 by gene expression (Repressor LexA).

Figure 2: Mechanism of action of isolated compound A1(Epigallocatechin, Epicatechin and stigmasterol phytosterol (Synergy) by gene expression (Repressor LexA) on Bacillus subtilis.
Figure 3: Mechanism of action of isolated compound A3(Aspidofractinine-3-methanol) by gene expression (Repressor LexA) on Bacillus subtilis.

Figure 4: Mechanism of action of isolated compound F3(Terephthalic acid, dodecyl 2-ethylhexyl ester) by gene expression (Repressor LexA) on Bacillus subtilis.

Target genes or Biomarkers

<table>
<thead>
<tr>
<th>Forward 5'-3'</th>
<th>Reverse 5'-3'</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GACTTGCTGGCAGTGCATAA</td>
<td>TCAGGCGCTTAACGGTAACT</td>
<td>Zehr et al. (2001)</td>
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Table 2: Primers used for PCR molecular investigation, to determine the mechanisms of action of novel compound isolated from ethyl acetate extract of Spondias mombin on selected clinical organisms.

Discussion

The purpose of the research work is to determine the mechanism of action of compound A1, A3 and F3 from the ethyl acetate stem bark extract of Spondias mombin. It was observed that isolated compounds A1, A3 and F3 has bactericidal action on E. coli at 180 minutes of administration using repressor LexA gene which stimulate the test organism to death phases. The compounds A1, A3 and F3 disrupt the SOS regulon without activation; the bacteria cells were complete destroyed. This is represented in Figures 5-8. Bactericidal action were observed at 1000 μg/mL as shown in Figures 5-8 in compound A1, A3 and F3 on E. coli.

Figure 5 shows the mechanism of action of isolated compounds A1, A3 and F3 from the ethyl acetate stem bark extract of Spondias mombin. It was observed that isolated compounds A1, A3 and F3 has bactericidal action on E. coli at 180 minutes of administration using repressor LexA gene which stimulate the test organism to death phases. The compounds A1, A3 and F3 disrupt the SOS regulon without activation; the bacteria cells were complete destroyed. This is represented in Figures 5-8. Bactericidal action were observed at 1000 μg/mL as shown in Figures 5-8 in compound A1, A3 and F3 on E. coli.

The death phase between 0 to 30 minutes, and a sharp decrease in death phase at 60 minutes but the organism survived at 180 minutes. The same activity was found in relative response of A1 LexA and F3 LexA to Bacillus subtilis.

In Figures 1-4, the mechanism of action of compound A1, A3 and F3 were demonstrated by Repressor LexA gene with Escherichia coli to demonstrate the sequential death phase of the organism between 0-180 minutes time interval. It was observed that A1 LexA shows a rapid increase in activity between 0 to 180 minutes and at 180 minutes, the organism rapidly enters the death phase, there is an increase in growth at 0 minutes and decrease in growth at 180 minutes i.e the compound completely inhibit the Escherichia coli at 180 minutes. The same activity was found in relative response of A3 LexA and F3 LexA to Bacillus subtilis.

In Figures 5-8, the mechanism of action of compound A1, A3 and F3 were demonstrated by Repressor LexA gene with Escherichia coli, to demonstrate the sequential death phase of the organism between 0-180 minutes time interval. It was observed that A1 LexA shows a rapid decrease in activity from 0 to 180 minutes and 180 minutes the organism rapidly enters the death phase, there is an increase in growth at 0 minutes and decrease in growth at 180 minutes i.e the compound completely inhibit the Escherichia coli at 180 minutes. The same activity was found in relative response of A1 LexA and F3 LexA to Bacillus subtilis.

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Figure 5: Agarose gel electrophoresis of the amplification of product coding LexA (620 bp) selected interaction between gene and cells (Ladder DN 100 bp). Mechanism of action of isolated compound A1, A3 and F3 by gene expression (Repressor LexA) on Escherichia coli.

Figure 6: Mechanism of action of isolated compound A1 (Epigallocatechin, Epicatechin and Stigmasterol phytosterol (Synergy)) by gene expression (Repressor LexA) on Escherichia coli.

In evaluating the pathway in Figure 1, the mechanism of LexA gene must be discussed for the clarity of purpose. In the presence of single stranded DNA, LexA interacts with RecA causing an autocatalytic cleavage which disrupts the DNA binding part of LexA, leading to depression of the SOS repair and eventually DNA repair auto cleavage is maximal at pH 11 in the absence of RecA and ssDNA, this activity leads to Ma-1-Gly bond in repressor LexA [21]. Erill et al. [7] also reported that repressor LexA is a transcriptional repressor that represses SOS response genes coding primarily for error prone DNA polymerase, DNA repair enzymes and cell division. LexA forms defacto, a two component regulatory system with RecA which senses DNA damage at stalled replication fork forming monofilaments and acquiring an active conformation capable of binding to LexA and causing LexA to cleave itself in a process called autoproteolysis [22].

Figure 7: Mechanism of action of isolated compound A3 (Aspidofractinine-3-methanol) by gene expression (Repressor LexA) on Escherichia coli.
In this research, it was discovered that the effect of the isolated compound from ethyl acetate Spondias mombin extract on the organism can be measured to death phase. If the isolated extract were introduced to the organism i.e Escherichia coli and Bacillus subtilis, it will serve as antibiotics which will cleave the DNA strands of the organism and there are possibilities that the damage is immense through bacteriostatic and bactericidal action. Before the LexA repairs the DNA, the death rate with time can be measured [23]. DNA damage can be created by the action of isolated compound, A1, A3, F3, antibiotics, etc. Ubeda et al. [3] reported that bacterial species such as Escherichia coli and Bacillus subtilis require topoisomerases such as DNA gyrase or topoisomerase IV for DNA replication. Isolated compounds A1, A3, and F3 under the SOS response increase the basal mutation rate of bacteria. Although mutations are often lethal to the cell, they can also enhance survival [24].

Figure 8: Mechanism of action of isolated compound F3(Terephthalic acid, dodecyl 2-ethylhexyl ester) by gene expression (Repressor LexA) on Escherichia coli.

In Figures 1 and 5, the bacteria (Escherichia coli and Bacillus subtilis) respond to the inclusion of isolated compound thereby leading to the DNA damage, mutation and death of the cell occur but late toward the death phase, the organism survived send a SOS repairs to their DNA, it is shown in the graph that the organism later survive the attack of the isolated compound but part of their DNA were lethally damaged, this account for the increase and decrease of death response with time in the graph in Figures 1-8 respectively. It should be noted that the effects of isolated compound leads to the death of the organism is possible because LexA contains a DNA binding domain where the isolated compound can attach itself. The winged HTH motif of LexA is a variant form of the helix-turn DNA binding motif which is usually located at the N-terminus of the protein [20], therefore the mechanism of action of compound A1, A3, and F3 is by damaging the DNA strands of the organism which leads to the death of the organism, this is demonstrated by the pathway [25].

Conclusion

In conclusion, the bacteriostatic and bactericidal mechanism of action of Epigallocatechin, Epicatechin and Stigmasterol phytosterol (Synergy), Aspidofrutinine-3-methanol and Terephthalic acid, dodecyl 2-ethylhexyl ester were measured by selective biomarker and gene expression, repressor LexA on Escherichia coli and Bacillus subtilis. It was observed that LexA gene increase a rapid progress of organisms to sequential dormancy and death phase with time interval, this led to the death of the bacteria cell, it can be deduced that, this method can be used to program the death of bacteria cell in case of a recalcitrant infections.

It is thereby recommended that the medicinal plant such as Spondias mombin and other types of medicinal plants should be studied and exploited for future use.

References