Bioguided search for anticancer natural products in the plant *Annona senegalensis*: a preliminary efficacy assessment

Abstract

*Annona senegalensis* is a plant widespread in tropical regions including the sub-saharian region of the African continent. Generally, all the different parts of the plant are used in traditional medicine. It had been previously reported to contain cytotoxic, antibacterial, antiparasitic and antibiotic compounds. In the present report, we have evaluated the antitumor activity of the *Annona senegalensis* plant in vitro by targeting with different extracts of this plant the ribosomal protein (rp) eL42 in the human 80S ribosomes in situ. In fact, rp eL42 had been shown to bind most of the anticancer drugs targeting the ribosomes. Since this protein had been found to be crosslinked in situ on human 80S ribosomes with periodate-oxidized tRNA, a reactive analogue of the tRNA molecule, we took advantage of the fact that any anticancer natural product targeting this protein would prevent the crosslinking reaction. Our results reveal that the crude extract, as well as the methanol and butanol fractions at 10μg/μl contain bioactive molecules that bind to the Lys-53 residue of the eL42 protein and prevent the crosslinking reaction with tRNAox.

Finally, *Annona senegalensis* was shown to exhibit a dose-dependent cytotoxic effect on breast cancer MDA-MB-231 cells. Altogether, the results in the present report suggest that *Annona senegalensis* is likely to contain a very effective molecule targeting the ribosomal protein eL42 with a potent anticancer effect on breast cancer cells in vitro.

**Keywords:** *Annona senegalensis*, human 80S ribosomes, ribosomal protein eL42, Lys-53 residue of rp eL42, periodate-oxidized tRNA, affinity labeling in situ of eL42 on 80S ribosomes, anticancer natural products

**Abbreviations:** ETMs, ethnomedicinal recipes; TMPs, traditional medicine practitioners; tRNAox, tRNA molecule;

**Introduction**

Medicinal plants are generally the primary source of new compounds used for healthcare in human. The World Health Organization (WHO) had indicated that 85 % of the world population uses the plants or their isolated bioactive compounds for healthcare. One well-known example is docetaxel (also named taxotere), an active molecule derived from paclitaxel which is extracted from the leaves of the European If plant (*Taxus baccata*). In this view, African herbalists possess indigenous ethnomedicinal recipes (ETMs) for the management of several diseases including cancer. The aim of the work reported here is to initiate a collaborative and preliminary sample study of african ethnomedicines used by the traditional medicine practitioners (TMPs), for the management of cancers, and to evaluate the scientific basis for the use of these traditional remedies. To this purpose, we have started to conduct a bioguided search for anticancer natural products in African plants by taking advantage of the recent demonstration that the large subunit ribosomal protein eL42 from human 80S ribosomes might be a candidate target for anticancer therapy. This protein exhibits the following characteristics:

1. It was recently shown to directly and actively contribute to the activity of 80S ribosomes at the elongation step of translation, and thus suggesting that this rp might control the rate of protein biosynthesis in health and in disease;
2. It was found to be overexpressed in human hepatocellular carcinoma as well as in several human tumor cell-lines, suggesting that its extra-ribosomal role might be related to tumor cell proliferation.
3. In the crystallographic structure of *S. cerevisiae* 80S ribosomes or of the 50S subunit of *Haloarcula marismortui*, most of the anticancer drugs were shown to target the eL42 protein.
4. We have recently demonstrated that rp eL42 might represent a therapeutic target in the human cancer-pertinent rp.eL42-p53-Mdm2 pathway.

In fact, the ribosomal protein eL42, the tumor suppressor protein p53 and the ubiquitin E3 ligase Mdm2 were shown to interact with each other in a ternary rp.eL42-p53-Mdm2 complex. In particular, the interaction between eL42 and p53 is characterized by a strong binding affinity (Kₐ value in the nanomolar range) that is likely to trigger the sequestration of p53 and the inhibition of its tumor suppressor activity, especially in the case of overexpression of eL42. Therefore, we had proposed that eL42 might be considered as a cancer promoter, while its overexpression might represent a direct cause of cancer formation through the downregulation of the tumor suppressor p53. First identification of rp eL42 had been achieved by affinity labeling this protein in situ on human 80S ribosomes by means of a reactive analogue of the tRNA molecule (tRNAox) obtained by the periodate oxidation of the tRNA.
treatment of native tRNA, which specifically oxidizes the 2',3'-cis-diol function of the 3'-terminal ribose of tRNA. Recently, tRNAox was found to be cross-linked with Lys-53 of eL42 in situ on human 80S ribosomes.⁹,¹⁰ Affinity labeling of rp eL42 by tRNAox consists in the formation of a reversible Schiff base between the 2',3'-aldehyde groups of tRNA and the ε-amino group of the Lys-53 residue. The equilibrium for Schiff base formation is displaced by reduction with sodium cyanoborohydride.¹¹ We have recently demonstrated that the small-molecule inhibitors such as thiosemicarbazones that bind to the amino group of the side chain of Lys-53 of eL42 are capable of inhibiting the crosslinking reaction between tRNAox and eL42 by preventing Schiff base formation.¹² Therefore, the presence in a plant extract of a small-molecule inhibitor targeting rp eL42 might be accounted for by the inhibition of the crosslinking reaction between tRNAox and eL42.

Materials and methods

Materials

Biological Materials

The biological materials used are: human 60S and 80S ribosomes, periodate-oxidized tRNA (tRNAox) and anti-eL42 antibodies.

Plant material

The leaves of the plant Annona senegalensis were used for the evaluation of biological activity (Figure 1).

Methods

The Annona senegalensis plant was selected in collaboration with 37 traditional medicine practitioners (TMPs) from 3 areas (Abomey, Bohicon and Agbangnizoun) of the Zou Department (Benin). Then, the leaves were layed out on the bench and allowed to dry for 3 days in a ventilated room at 16°C. Finally, the leaves were pulverized, before the addition of water (decoction) or ethanol (maceration).

Preparation of extracts

The decoction or maceration

In the present work, decoction was performed at 10% by mixing 100g of the plant powder and 1L of distilled water in a pyrex balloon (2L). The mixture was boiled for 15min. Maceration consisted in mixing 100g of medicinal plant powder and 1L of ethanol 96% during 48 h with shaking. Then, the decoction or the maceration mixtures were filtered on a paper or a cotton filter, depending on the hydrophilicity of the sample, and rinsed with a small volume of hot distilled water to obtain 800mL of filtrate. Both filtrates representing complete mixtures were evaporated at 35°C in a Rotavapor system and referred to as the crude extracts of the plant Annona senegalensis. These crude extracts were further fractionated with methanol or butanol.

Phytochemical screening of Annona senegalensis

The phytochemical screening of the Annona senegalensis powder was performed according to the method of Houghton & Rama¹³ which is an analytical method of characterization of the main groups of chemical compounds such as tannins, polyphenols, flavonoids, quinones, triterpenes, steroids, alkaloids, etc in medicinal plants (Table 1). It is based on the specific detection and/or precipitation of these molecules. The plant Annona senegalensis was found to contain only slight amounts of free anthracene and C-glycosides (Figure 2).

Table 1 Chemical characterization of the molecules present in the plant Annona senegalensis

<table>
<thead>
<tr>
<th>Chemical Groups</th>
<th>Subgroups</th>
<th>Observations</th>
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<tbody>
<tr>
<td>Tannins</td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>Flavonoids</td>
<td></td>
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<tr>
<td>Anthocyanins</td>
<td></td>
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<td>Compounds polyphenolics</td>
<td>Leucanthocyanins</td>
<td>-</td>
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<td>Alkaloids</td>
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<td>++</td>
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<tr>
<td>Reducing compound</td>
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<td>+++</td>
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<tr>
<td>Quinone derivatives</td>
<td></td>
<td>-</td>
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<tr>
<td>Mucilages</td>
<td></td>
<td>+++</td>
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<tr>
<td>Cyanogenic derivatives</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Cardiotonic derivatives</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Anthracene derivatives</td>
<td>C-glycosides</td>
<td>++</td>
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<tr>
<td>Anthracene derivatives</td>
<td>O-hétérosides</td>
<td>-</td>
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<tr>
<td>Triterpenes</td>
<td></td>
<td>-</td>
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<tr>
<td>Steroids</td>
<td></td>
<td>+++</td>
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<tr>
<td>O-glycosides</td>
<td></td>
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</table>

Chemical characterization of the molecules present in the plant Annona senegalensis. (-), absent; (+), slight amount; (++), medium; (+++), high amount
The solvents (water and ethanol) made it possible to extract many constituents of the leaves and roots of the tested plant. Phytochemical studies have revealed the presence of numerous constituents such as reducing compounds, tannins, flavonoids, mucilages, monosaccharides and oligosaccharides, steroids, anthocyanins and alkaloids. For the evaluation of the pharmacological properties related to the therapeutic indication, water seems to be a better solvent for the extraction of the majority of the chemical constituents responsible for the various antibacterial and antifungal activities of the plants justifying the relevance of the traditional form of use by the traditional medicine practitioners (TMPs). The presence of bioactive molecules in the *Annona senegalensis* plant had been previously reported.\(^\text{14,15}\)

**tRNAox-labeling reaction**

Periodate-oxidized tRNA (tRNAox) was prepared as described previously.\(^\text{9}\) The principle of the covalent labeling is to use a reactive tRNA analogue directed to the catalytic site of purified enzymes within the translation apparatus, or to the A, P and E-sites of the ribosome. This analogue is periodate-oxidized tRNA (tRNAox), the dialdehyde derivative of tRNA whose 2',3' cis-diol group of the ribose of the 3'-terminal adenose has been converted to a 2',3' dialdehyde group by sodium periodate (Figure 3).\(^\text{9–11}\)

![Figure 3 Periodate-oxidized tRNA.](image-url)
The aldehyde function at the 2’ or 3’ position of tRNAox is capable of forming a Schiff base with the side chain of a lysyl or an arginyl residue. The Schiff base is then stabilized by a specific reducing agent, sodium cyanoborohydride (NaBH₄CN) to allow the formation of a covalent complex between the tRNA molecule and a lysyl residue of the protein (Figure 4). When [³²P]tRNAox was used, the covalent complex was detected by autoradiography. tRNAox-labeling with non radioactive tRNAox molecules was initiated by the addition of non radioactive tRNAox (100μM) in a 10μL reaction mixture. The mixture was also composed of 1μL of 50mM sodium cyanoborohydride (NaBH₄CN) and 1μL of 10X buffer (labeling buffer 10 times more concentrated). After 1h of incubation at 37°C, the mixtures were treated by adding 1.5μL of 0.5M sodium borohydride (NaBH₄) freshly prepared in water (DNase/RNase Free) and 3.5μL of Laemmli 3X. 15μL portions of the samples treated with NaBH₄ were analyzed by electrophoresis on 8%, 10% or 12% polyacrylamide gel (depending on the molecular weight of the protein) under denaturing conditions (SDS-PAGE). The gels were revealed by staining with coomassie blue or by western blotting (Figure 4).

Figure 4 tRNAox-labeling reaction.
Cell culture

Breast cancer, MDA-MB-231 cells were a generous gift from Professor Gérard Perret (Avicenne Hospital, Bobigny, France). They were cultured in liquid medium; Dulbecco’s Modified Eagle Medium 1X (DMEM) (Sigma Aldrich) supplemented with 10% Foetal Bovine Serum, FBS, (Sigma Aldrich) and 1% non essential amino acids. The cells were cultured in an incubator set at 37°C with 5% CO₂ and 85% humidity.

Results and discussion

Affinity labeling in situ of rp eL42 with tRNAox on the human 80S ribosomes

A prerequisite for the search for small-molecule inhibitors of the crosslinking reaction in extracts of the Annona senegalensis is to verify that the large subunit ribosomal protein eL42 can be effectively crosslinked in situ with tRNAox on human 80S ribosomes as previously reported by our group.16 For this purpose, we have followed the tRNAox-labeling reaction between the human 80S ribosomes and [32P]tRNAox as a function of the pH of the incubation mixture. Interestingly, the incorporation of [32P]tRNAox into eL42 inside the human 80S ribosomes was shown to increase as a function of increasing pH values (Figure 5). This result reflects the dissociation of the protonated form of the ε-amino group of Lys-53 and its greater reactivity through the tRNAox-labeling reaction, in accordance with previously reported data.16,17

Fig. 5 shows the autoradiogram of the labeling of the eL42 protein within the human 80S ribosome by tRNAox, analyzed by electrophoresis on a 10% polyacrylamide gel in urea (Urea-PAGE).

Affinity labeling in situ of rp eL42 with tRNAox on the human 80S ribosomes in the presence of Annona senegalensis extracts

The effect of extracts of Annona senegalensis on the crosslinking reaction was checked as follows: human 80S ribosomes were incubated with tRNAox and Annona senegalensis plant extracts, followed by analysis by electrophoresis on a 10% polyacrylamide gel under denaturing conditions (SDS-PAGE) revealed by anti-eL42 antibodies (Western Blot). As shown in Figure 6, incubation of 80S ribosomes in the presence of tRNAox gives rise to the formation of an eL42-tRNAox covalent complex (Lane 2 in Figures 6A–6C), as previously described. As expected, the 37 kDa band (Lane 2 in Figures 6A–6C) corresponds to the formation of a covalent complex containing one molecule of endogenous human eL42 (12,000Da) and one molecule of tRNAox (25,000 Da; calculated MW of the eL42-tRNAox complex 37,000 Da). By contrast, the absence of a 37 kDa band in Lanes 3 (Figures 6A & 6B) suggests that the methanol and butanol fractions at 10 μg/μL contain molecules that would prevent the formation of the covalent eL42-tRNAox complex. Therefore, it is most likely that the Annona senegalensis plant contains molecules capable of binding to the Lys-53 residue of rp eL42 leading to a competition with the covalent binding of the tRNAox molecule. Lane 1 in Figs. 6A, 6B and 6C represents the control 80S ribosomes alone (i.e. in the absence of tRNAox) where no cross-linked eL42 band was visible, while only the eL42 band revealed by the anti-eL42 antibodies was present. Another control experiment where 80S ribosomes were incubated with tRNAox in the presence of the crude extract of Annona senegalensis in water gave the same result as in Lanes 3 (Figures 6A & 6B). Finally, we have checked the effect on the tRNAox-labeling of eL42 in situ on human 80S ribosomes of 4-phenyl-3-thiosemicarbazone piperitone which had been previously shown to prevent tRNAox from binding to Lys-53 of rp eL42 (Lane 3 in Figure 6C). Altogether, the data shown in the present report strongly support the presence in the Annona senegalensis plant of molecules capable of binding to rp eL42, similarly to the majority of the anticancer drugs targeting the eukaryal or archael ribosomes.

Effect of the crude extract of *Annona senegalensis* on breast cancer MDA-MB-231 cells

Fig. 7 shows a dose-dependent response of breast cancer MDA-MB-231 cells to treatment with a crude extract of *Annona senegalensis* by demonstration of increased cytotoxicity. Therefore, this result suggests that *Annona senegalensis* is likely to contain a very effective molecule for the eradication of breast cancer MDA-MB-231 cells *in vitro* (Figure 7).

![Image](image_url)

**Figure 7** Effect of the crude extract of *Annona senegalensis* on breast cancer MDA-MB-231 cells. MDA-MB-231 cells were incubated during 24 hours in the absence (A) or the presence of the crude extract of *Annona senegalensis* in water at 5μg/μL (B), 10μg/μL (C) or 15μg/μL (D). An inverted light microscope (Nikon TMS-F 5.2 dia x 20 mm) with a built in camera was used to capture images of the cells. 10 × magnifications were used to view the morphological appearance of the cells.

Conclusion

In this report, we have evaluated the antitumor activity of the *Annona senegalensis* plant *in vitro* by targeting with different extracts of this plant the eL42 protein in the human 80S ribosomes *in situ*. In fact, we took advantage from the fact that rp eL42 had been shown to bind most of the anticancer drugs targeting the ribosomes, and that this protein had been found to be crosslinked *in situ* on human 80S ribosomes with periodate-oxidized tRNA, a reactive analogue of the RNA molecule. Our results reveal that the crude extract, as well as the methanol and butanol fractions at 10μg/μL contain bioactive molecules that bind to the Lys-53 residue of the eL42 protein and prevent the crosslinking reaction with tRNAox. In addition, *Annona senegalensis* was shown to exhibit a dose-dependent cytotoxic effect on breast cancer MDA-MB-231 cells. Altogether, the results in the present report suggest that *Annona senegalensis* is likely to contain a very effective molecule targeting the ribosomal protein eL42 with a potent anticancer effect on breast cancer cells *in vitro*.

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None.

Conflicts of interest

The authors declare that there is no conflict of interest.

References
