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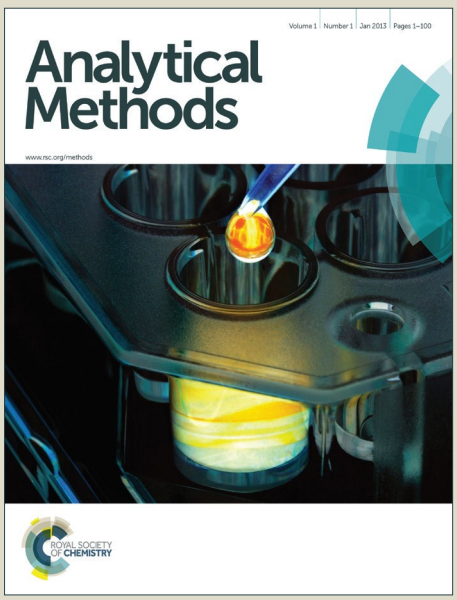

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DNA-EB in agarose gel assay: a simple methodology in the search of DNA-binders in crude extracts from actinomycetes

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ABSTRACT

DNA is known as a potential therapeutic target for development of anticancer and antibiotic agents, where any damage on its structure may lead to cell death. Discovery of ligand molecules through interaction with DNA is a key step in the development of new therapies. Here, it was proposed a methodology to detect DNA-binders in a screen process with crude extracts produced by actinomycete. This assay consists of a DNA-EB (ethidium bromide) complex added previously, in a simple agarose gel, where the EB may be replaced by other metabolite, present in the sample added before over the gel. The free dye is washed and, consequently, the fluorescence intensity decrease.

The assay results were recorded by photographs and the fluorescence intensity was transposed in pixels values using Image J software, allowing statistical analyses. The developed assay can also be applied directly onto TLC plates. It was screened sixteen crude extracts produced by actinomycetes using the developed assay, in which four crude extracts showed significant fluorescence decrease. Dereplication study with mass spectra data allowed the identification of anthracyclines such as Ditrisarubicin F and G in one of the selected extract. The methodology developed here is a promising approach to drug discovery, because it may lead to detect DNA-binding compounds in a simple, with low cost way and without isolation and/or purification processes.

Keywords: DNA-binding; Mass spectrometry; Thin layer chromatography; Image J software; Ethidium bromide; Agarose gel.

Introduction

DNA is known as a potential therapeutic target because any damage on its chain may affect DNA replication leading to cell death. Anticancer and antibiotic agents studies, in which their biological activity is related to DNA interaction.[1, 2] There are many ways in which a small molecule can interact to DNA affecting its structure and function.[3] For instance, the planar ring systems of anthracyclines (known as anticancer compounds) intercalate between DNA base pairs; and their sugar(s) interact(s) with the minor groove.[4, 5] Another example is ethidium bromide, an organic dye very used in molecular biology, also intercalates between DNA base pairs.[6]

Microbial sources are well known as an important provider of a large diversity of pharmacotherapeutic compounds, and they have extensively been explored to drug discovery researches.[7] However, the chemical diversity of secondary metabolites challenges the processes of bioactive compounds isolation and purification. Thus, screening strategies may be more attractive to apply in natural products to guide and facilitate the identification of novel biological activity compounds. A variety of physicochemical and biochemical techniques have been applied to screen and study DNA-binding complexes, such as: Raman;[8] agarose gel electrophoresis;[9] resonance light scattering (RLS); ultraviolet spectra (UV) and ^{1}H NMR spectroscopy;[10] and others. On-line combination of chromatographic, mass spectrometry and fluorescence techniques have also been employed efficiently, to obtain HPLC fingerprint, UV spectra, MS^{n} fragments and DNA-binding profile of crude extracts, an approach to save time.[11, 12] Nevertheless, those methodologies are expensive to be used to a large amount of sample (e.g. screening processes), needing sophisticated equipments and laboratories.

In this work, two simple, low cost and rapid approaches were used in an assay to screen *in vitro* compounds with interactions to DNA. The methodology presented here was very suitable to detect DNA ligand compounds present in the crude extract produced by actinomycete CS039. Dereplication for the active crude extract employing tandem mass spectrometry, allowed the identification of Ditrisarubicin F and G, both belonging to anthracyclines class.

Material and methods

Chemicals

Agarose was bought from Agargen. DNA from herring sperm, ethidium bromide, safranin T, doxorubicin (Dox), daunorubicin (Dnr), colchicine (Colch), combretastatin A-4 (CA-4), vinblastine (Vinblast), camptothecin (CPT) were purchased from Sigma-Aldrich. Gel Red Nucleic Acid Stain was obtained from Biotium. All the above compounds were used without further purification. Dox and Dnr stock solutions were prepared in HPLC grade methanol (MeOH) at 200 μg/mL (w/v). DNA stock solution was prepared in ultra-pure water at 1 mg/mL (w/v), and ethidium bromide (EB) and safranin T (Sf_T) stock solutions were prepared in HPLC grade MeOH at 20 μM. A DNA:EB mixture was prepared adding 50 % of each, leading to a final concentration at DNA 0.5 mg/mL and EB 10 μM. The DNA concentration was determined according to absorbance at 260 nm after establishing the absorbance ratio, A_{260}/A_{280} to be in the range of 1.80–1.90. This indicated that the DNA was free from protein.

Microorganisms

Actinomycetes were kindly provided from *Coleção de Microrganismos de Importância Agrícola e Ambiental* (CCMA, EMBRAPA, Brazil). The strains were maintained as mycelia fragments in glycerol (20 %, v/v) at -20 ^{o}C, and reactivated on plates of GYEA (glucose-yeast extract agar)[13] at room temperature.

Crude extracts

Actinomycetes were cultivated in 125 mL Erlenmeyer flasks containing 25 mL PD (potato-dextrose) medium at 30 °C under shaking conditions (150 rpm) for 10 days. Crude extracts were obtained by simple liquid culture filtration followed by liquid-liquid extraction with ethyl acetate. The organic phase was dried and the crude

extracts obtained were diluted with MeOH at final concentration 10.0 mg/mL, and the solution was stored at 4 °C.

SAG – Simple Agarose Gel

Agarose 1.0 % was dissolved in ultra-pure water, heated in microwave oven, and poured into a 8x12 cm plastic plate. After solidify, 2 μL DNA:EB mixture was pipetted on the gel surface in different spots. Samples (2 μL) were pipetted exactly in the same spot where the DNA-EB mixture was previously pipetted.

G-DNA-EB – Gel-DNA-Ethidium Bromide

Agarose 1.0 % was dissolved in ultra-pure water, heated in microwave oven, and before solidify, different percentages of DNA:EB mixture were added into the solution (5-25%). Samples were pipetted in different spot on the gel surface.

DNA-Binding Assay optimization

To develop this assay the following variables were evaluated: agarose concentration, sample volume pipetted over the gel, extracts concentration (10, 1.0 and 0.1 mg/mL), EB displacement time (20, 30, 60 and 120 minutes) and washing time (10, 20, 30 and 60 minutes).

All experiments were developed in triplicate. After pipetting samples on the gels, both gel types (SAG and G-DNA-EB) were left for 30 minutes to sample permeation, and then submerged in ultra-pure water and kept under shaking conditions (80 rpm) for 20 minutes. The gels were photographed on fluorescent transilluminator [302 nm wavelength (λ)]. The images were processed by Image J software[14] and pixel values were considered for each point to statistical analyses.

The method specificity was evaluated using Safranin T (Sn_T) and Gel-red (GR), at 20 μM, mixture to DNA 1 mg/mL, before being pipetted on SAG surface, and after 30 minutes Dox was pipetted.

The solvent effect was also evaluated in the developed assay and the following were tested: DMSO (dimethyl-sulfoxide), MeOH (methanol), EtOH (ethanol), DMF (N,N-dimethyl-formamide), ACN (acetonitrile), acetone, ethyl acetate, chloroform and hexane.

To evaluate the sensitivity for the developed assay, two compounds Dox and Dnr, known as DNA-binders, were used, at 200, 100, 50, 10, 1.0 and 0.1 μg/mL dissolved in MeOH.

The method was evaluated using anticancer standards with different mode of actions: doxorubicin, daunorubicin, colchicine, combretastatin A-4, vinblastine and camptothecin. All of them were tested at 200 μg/mL. Vinblastine was dissolved in DMSO and the other standards in MeOH.

Crude extracts obtained from actinomycete were submitted to the DNA-binding developed assay, applying 2 μL of each, only on SAG gel.

Mass spectrometry analyses

Mass spectrometry analyses (full scan and CID – Collision Induced Dissociation) were developed employing an Acquity™ UHPLC system (Waters®) consisting by a quaternary pump and automatic injector coupled to Xevo TQ-S mass spectrometer with a Z-spray orthogonal ionization source ESI (electrospray) (Waters®). CID experiments were performed in positive ionization mode (ESI^+), and the MS parameters were as follows: 3.2 kV capillary voltage, 40 V skimmer voltage, 300 °C dessolvation chamber temperature, 600 L/h gas flow, 5.0 Bar nebulizer gas, and 30 eV collision energy. To

data acquisition and processing, Masslynx 4.1 software (Waters Corp., Milford, MA, USA) was used.

Statistical analyses

The data obtained were analyzed in GraphPad Prism 5.0 software using ANOVA and Tukey test. It was considered 5.0 % significance ($p < 0.05$ value).

Results and Discussion

DNA offers a number of potential binding sites and modes of noncovalent interactions for small molecules. Many organic dyes have already proven to be sensitive probes to DNA, such as ethidium bromide (EB),[15, 16] acridine orange (AO),[17] diphenylamine blue[18] and neutral red,[19] among others. These organic dyes have a planar aromatic system that can insert between two adjacent base pairs in a helix and incorporate a positive charge.

A simple, fast and low coast DNA-binding assay was developed, optimized and used to screen crude extracts produced by Brazilian actinomycetes. The DNA-binding assay was developed to reduce the number of sample and focused the search for DNA binders in crude extracts. The focus of this work was the development of a simple assay to detect DNA binders as promisor anticancer in complex samples as microbial crude extracts. The DNA-binding assay developed is a simple method that could be applied in many laboratories, without necessary high technologies; in other words, this approach is appropriate to detect DNA-binders. For identification it is necessary other techniques as liquid chromatography, mass spectrometry, nuclear magnetic resonance and others.

Although, the developed method helps guiding and reduces the number of samples to be analyzed by advanced techniques.

The DNA-binding assay uses agarose gel as a support to DNA in a way that the compounds could be in contact with the DNA and those did not bounded could be released with washing. The organic dyes bonded in DNA could be substituted by another binder, this replacement occurs due to secondary metabolites presence in the crude extracts with higher affinity to DNA. The organic dye released could be eliminated by washing, and the positive results show that the fluorescence spot intensity was gradually decreased according to organic dye release.

In this work, it was developed two different methods with the same principle: SAG and *G-DNA-EB*. At beginning, it was developed and optimized the (**1**) SAG methodology to use low amounts of sample. After that, the SAG gel was changed, developing the (**2**) *G-DNA-EB,* making it possible to be applied directly onto TLC plates, to guide de isolation of the compounds detected as DNA binders. To develop the DNA-binding assay, it was used a low cost DNA in agarose gel, and Doxorubicin, a well know DNA binder, to evaluated if the idea works. For these, various conditions including the agarose gel concentration, the DNA and EB concentration on gel, EB displacement time and the Dox concentration were investigated (data not showed). Finally, SAG was applied to screen sixteen crude extract leading to detection of four sample containing promisor anticancer compounds. Mass spectrometry allowed the identification of Ditrisarubicin D and G, in one of the crude extracts. These compounds belong to a class of anthracyclines, known as DNA-binding.

(1) SAG methodology

a) DNA-binding assay specificity

It was evaluated the effect of different organic dyes rather than ethidium bromide (EB), such as Gel-Red (GR) and Safranin T (Sn_T), which are compounds less dangerous and have similar performances, on fluorescence spot intensity.[20] It was prepared solutions adding 50 % of each (DNA + one organic dye), leading to a final concentration at DNA 0.5 mg/mL and organic dye 10 μM. It was prepared a solution for each organic dye separated. 2 μL of the solution DNA + organic dye was pipetted onto the gel surface and hold until the drop was completely absorbed by the gel, approximately 15 minutes (Figure 1A). 2 μL Dox 200 μg/mL was added exactly on the same spot pipetted previously, and hold until the drop was completely absorbed by the gel and interact with DNA, approximately 30 minutes. It is necessary more time to organic dye, and other compounds to interact with DNA, however, it was observed that more than 40 minutes disperse the DNA and the spot fluorescence cannot be measured any longer. The same effect was observed for washing for more than 20 minutes. Figure 1B shows the fluorescence intensity after washing by 20 min.

Ethidium bromide is suitable as a fluorescent marker of nucleic acids because binds to DNA, and for this reason it is the most commonly used in molecular biology laboratories. Ethidium bromide (DNA+EB+Dox) proved to be the best organic dye to be used in this method (Fig. 1B), presenting a fluorescence intensity decrease of 56 % relatively to the control (DNA+EB). Gel-Red showed fluorescence intensity decrease of 41 % relatively to the control, which shows that the DNA-binding assay can be used with other safer markers than ethidium bromide, as Gel-Red. In contrast, no fluorescence was observed with Safranin T and this could be due to the wavelength (302 nm) used by the Transilluminator. Safranin T ideal wavelength absorbance is 520 nm.

b) Solvent effect

Crude extracts from natural products, or even pure compounds, are usually dissolved in a wide variety of organic solvents, as MeOH, acetonitrile, DMSO and others. Therefore, the organic solvent effect was also investigated if could cause interference in the fluorescence spot intensity (Supplementary material Table S1). N,N-Dimethyl-formamide (DMF) caused a little decrease in the fluorescence intensity compared with the other solvents, but it was not a significant decrease. This result means that the organic solvents tested can be used in this DNA-binding assay without influencing the fluorescence spot intensity.

c) DNA-binding assay sensitivity

To test the sensitivity for the SAG method, Dox and Dnr in different concentrations were used (from 200 to 0.1 ng/μL) (Table 1). This experiment permitted to observe significant statistical changes in fluorescence intensity at concentrations 200, 100, 50 and 10 ng/μL, for both Dox and Dnr. At below concentrations (1 and 0.1 ng/μL) no significant variation was observed (Table 1). It is important to note that Dox was used as a purified compound, and crude extracts are often used at higher concentrations since they are a mixture of compounds.

d) DNA-binding assay selectivity

The method selectivity was evaluated using known anticancer standards with different mode of actions: DNA-binders (Doxorubicin and Daunorubicin), tubulin-binders (Colchicine, Combretastatin-A4 and Vinblastine)[21] and topoisomerase-binder (Camptothecin).[22] As expected, only DNA-binders Dox and Dnr exhibited statistically significant decrease in fluorescent intensity, ~59 % and ~57 %, respectively. None of the other standards used with different modes of actions showed significant changes in fluorescent intensity (Fig. 2); in other words, these compounds did not interact with DNA in the DNA-binding assay, as expected, once their mode of action is binding to

other targets. This result demonstrates that the developed method can be used in DNA-binders detection. Camptothecin (CPT), a fluorescent compound (excitation: $\lambda_{máx}$ 370 nm; emission: $\lambda_{máx}$ 450 nm),[23] increased the fluorescence intensity, nevertheless, this increase was not statistically significant. It is important to note that compounds which are able to replace EB in DNA, but also have the same fluorescence emission range, can lead to a false negative result. Thus, in this kind of study, it is also necessary to evaluate the crude extract fluorescence to exclude any false-negative results.

e) Screening of crude extracts by SAG methodology

Once optimized the experimental conditions for SAG methodology, it was applied in a screen process with sixteen crude extracts produced by actinomycetes (Table 2). It was observed less intensity in fluorescence for crude extracts CS039 (~76%), SB074 (~74%), SB003R (~84%) and SB019 (~94%), and Dox (~49%) when they were compared with control DNA+EB (100%). These results suggest that the selected extracts present DNA-binding compounds in their compositions. Thus, the selected crude extracts were analyzed by mass spectrometry and dereplication study.

Several methodologies have been described in literature to study the interaction of DNA and binders, such as: mass spectrometry,[24] NMR, UV[10] and circular dichroism,[25] among others. A methodology established by Song et al.,[26] allows detection of DNA-binders on-line, combining techniques, such as HPLC, DAD (Diode Array Detector) and high resolution mass spectrometry. Nevertheless, all these methodologies require high technology equipment and specialized workforce. Besides that, these kinds of analysis are expensive, which makes their application impracticable, in many laboratories, in studies with large amounts of sample. This way, the methodology

developed here is really good to be used in screening processes, since it can reduce the number of samples to be analyzed by these techniques.

(2) G-DNA-EB methodology

In laboratories which work with natural products is common to start projects with crude extracts. This kind of sample uses to be complex and they are initially analyzed by Thin Layer Chromatography (TLC), to obtain chemical and physical characteristics about the compounds present in the crude extracts. It would be impressive if there was a way to detect directly on TLC plates which compounds are able to bind to DNA, facilitating the isolation process. Thus, it was also proposed a second assay employing the G-DNA-EB methodology, which could be applied directly onto TLC plates.

Differently from SAG methodology, where the mixture DNA:EB was pipetted on agarose gel to form a fluorescent spot; in the G-DNA-EB methodology the gel was prepared by adding DNA:EB mixture into the gel, before gelling, forming a fluorescent gel.

a) G-DNA-EB preparation

It was evaluated different concentrations of the mixture DNA:EB [5, 10, 15 and 20 % (v/v)] into the gel. Data showed [Supplementary material Fig. S1 (A)] that the gel fluorescent intensity increased proportionately with the increase in the DNA:EB concentration, as expected. In the same gels, 2 µL Dox 20 µM were pipetted, in duplicate [Fig. S1 (B)]. The positive results are observed by decrease fluorescence in the spot where Dox solution was added. The G-DNA-EB with DNA:EB mixture 5% (v/v) was chosen to follow the experiments due to high contrast.

b) *G-DNA-EB onto TLC plate*

To analyze if the DNA-binder (Dox) could migrate from TLC to the G-DNA-EB, it was used a TLC with Dox pipetted previously in different concentrations. Then, the G-DNA-EB gel was put in contact to the TLC. The time to keep them in contact was also evaluated and 1 hour was chosen due to being the best time for Dox to migrate from the TLC plate and forming a defined dark spot in the fluorescent gel (Fig. 3).

It is possible to observe in Fig. 3B, the gel fluorescent intensity decrease referring to the Dox spots on TLC plate in Fig. 3A. This result means that Dox was able to migrate from TLC to G-DNA-EB gel. This experiment proves the importance for this kind of gel development, allowing its use routinely in laboratories to rapid detection of DNA binders from complex mixtures like microbial crude extracts.

Natural products screening processes normally begin with a great amount of samples, and for this reason, methodologies with low cost and fast response are really desired. The methodology developed in this work is simple, easy to apply, and mainly with low cost, making it extremely interesting in screening process for a large number of natural products extracts.

Anthracyclines identified in crude extract produced by actinomycete CS039

Crude extract produced by actinomycete CS039 was active in the DNA-binding assay developed in this work, for this reason, this extract was submitted to dereplication study employing the data obtained from full scan MS (Fig. S2), CID experiments and further physic characteristics and biological source were also used to search in Dictionary of Natural Products. The search results suggest that anthracycline class was found. In ESI^+ full scan spectrum, it can be observed interesting ions group, in the *m/z* 570 to 610 range (ions *m/z* 577, 585, 593, 601 and 609). The *m/z* difference of 8 units between then, suggest that they are double-charged $[M+2H]^{2+}$. Thus, the ions with

lower intensity *m/z* 1153, 1169, 1185, 1201 and 1217 are referred to the same compounds with only one charge $[M+H]^+$.

To additional structural information, the ions *m/z* 1169 and 1185 were fragmented by CID (Supplementary material Fig. S2, and Fig. 4). The ion *m/z* 1185 loses 244 Da (*m/z* 941), followed by loss of 240 Da (*m/z* 701), referring to two different di-glycosides. Ion *m/z* 701 refers to the aglycone with two aminoglycosides (rhodosamine) at C-7 and C-10 positions. The loss of these two aminoglycosides, with consecutive water loss, leads to the formation of the ions *m/z* 544 and *m/z* 369. The ion *m/z* 158 was attributed to rhodosamine protonated. The ion *m/z* 1185 was assigned to Ditrisarubicin G (Fig. 4). The CID spectra for the ion *m/z* 1169 presents the same fragmentation pattern observed to Ditrisarubicin G. This fragmentation pattern and molecular weight was also possible to confirm the presence of Ditrisarubicin F in crude extract produced by actinomycete CS039.

Ditrisarubicins are anthracyclines with anticancer activity produced by *Streptomyces cyaneus* MG344-hF4.[27] Differences on structure and on sugar numbers have been reported by causing different effects in tumor cells.[24, 28]

Conclusion

In summary, two new methodologies were successfully developed to DNA-binding studies for simple and fast compounds monitoring with potential to interact to DNA, employing agarose gel. The sensitivity was evaluated and Doxorubicin was detected in both gels even at a 10 μg/mL concentration. This assay was optimized and the obtained results showed that this new assay can be used to screen a great amount of extracts in a

cheap and efficient way, besides its use in modes of action studies. Natural product sources are well known as an important provider of a large diversity of pharmacotherapeutic compounds, in other words, they have extensively been explored to drug discovery research.[7] However, the chemical diversity of secondary metabolites challenges the processes of bioactive compounds isolation and purification. Thus, screening strategies may be more attractive to apply in natural products to guide and facilitate the identification of novel biological activity compounds. Natural products screening processes normally begin with a great amount of samples, and for this reason, methodologies with low cost and fast response are really desired.

Since the developed assay is based on fluorescent intensity changes, false-negative results may be obtained by DNA-binding compounds with fluorescence. This methodology allowed detecting compounds known as DNA-binders, in a crude extract produced by actinomycetes. Dereplication process tandem mass spectrometry allowed the identification of the anthracyclines Ditrisarubicin F and G in crude extracts from actinomycete CS039.

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Figure captions

Fig. 1. Specificity study in SAG gel. (A) Sample arrangements using the DNA-binders EB, Sf_T and Gel-Red; (B) SAG gel after sample application and wash.

Fig. 2. Method selectivity evaluation using anticancer standards with different targets: DNA, tubulin and topoisomerase-binders. DNA-EB was pipetted the same way as the control and then the anticancer standards were applied over the spots (triplicate) ($p < 0.05$ value).

Fig. 3. (A) TLC plate with Dox pipetted in different concentrations (from 400 to 10 μg/mL). (B) G-DNA-EB gel after application onto TLC with different Dox concentrations for 1 hour. The gel was washed for 20 minutes.

Fig. 4. The fragment pathway proposed to Ditrisarubicin G (*m/z* 1185), an anthracycline with two trisaccharide chains bound to the carbons C-7 and C-10, identified in crude extract produced by actinomycete CS039; and the MS/MS spectrum.

Table 1

Average fluorescence intensity (%) for Dox and Dnr in different concentrations (200 to 0.1 ng/μL) in DNA-binding assay ($p < 0.05$ value).

Sample	Conc. ng/μL	Average %	SD	Significance
Control		100	3.4	---
Dox	200	44.2	3.6	Yes ***
	100	55.1	3.0	Yes ***
	50	58.8	2.8	Yes ***
	10	74.7	2.7	Yes ***
	1	90.3	2.7	No
	0.1	103.2	2.8	No
Dnr	200	71.5	1.3	Yes ***
	100	79.9	2.0	Yes ***
	50	82.7	4.7	Yes ***
	10	84.7	6.1	Yes ***
	1	93.3	3.9	No
	0.1	109.2	0.6	No

*** high significance

Table 2

Average fluorescence intensity for different extracts produced by actinomycetes applied in the developed DNA-binding assay ($p < 0.05$ value).

Sample	Average %	SD	Significance
Control	100	1.4	
Dox	49.0	0.3	Yes***
IR056	94.5	1.5	No
IRD009	98.5	1.5	No
SBA013	101.5	1.3	No
IR048	99.9	1.0	No
CS027	99.8	1.5	No
CS045	98.8	2.2	No
CS039	75.8	0.6	Yes***
SB019	93.5	2.2	Yes*
CS029	100.6	0.6	No
SB074	74.0	1.0	Yes***
IR064	94.1	2.4	No
SB028	96.5	1.2	No
SBE003A	95.2	3.7	No
SB003R	84.2	0.4	Yes***
SBE002	98.4	1.5	No
SB086	98.0	2.2	No

* low significance; *** high significance

Figure Graphics

Fig. 1.

A)

DNA+EB Control	DNA+EB +Dox	DNA+ Dox
DNA+GR Control	DNA+GR +Dox	DNA+ Dox
DNA+Sf_T Control	DNA+Sf_T +Dox	DNA+ Dox

B)

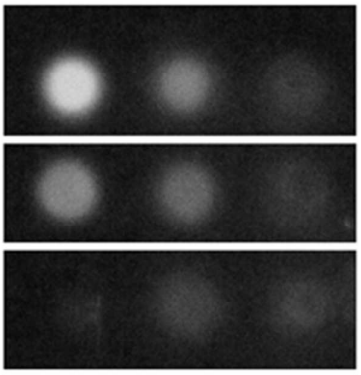
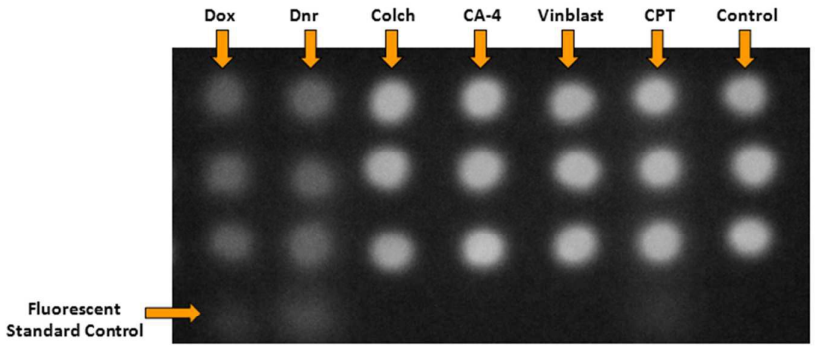


Fig. 2.



Sample	Average %	SD	Significance
Control	100.0	3.0	---
Dox	41.3	2.4	Yes ***
Dnr	43.4	3.7	Yes ***
Colch	100.2	4.9	No
CA-4	104.9	1.7	No
Vinblast	104.1	2.3	No
CPT	106.6	2.1	No

*** high significance

Fig. 3.

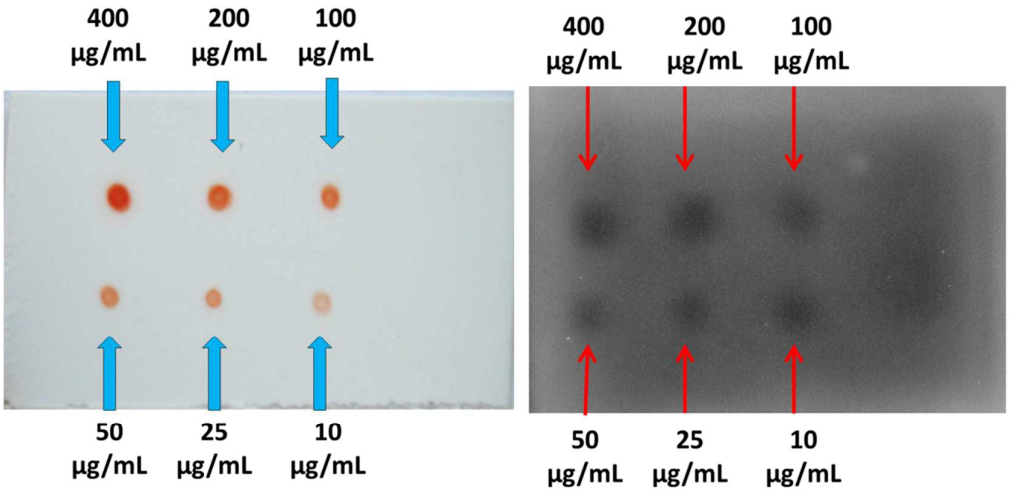
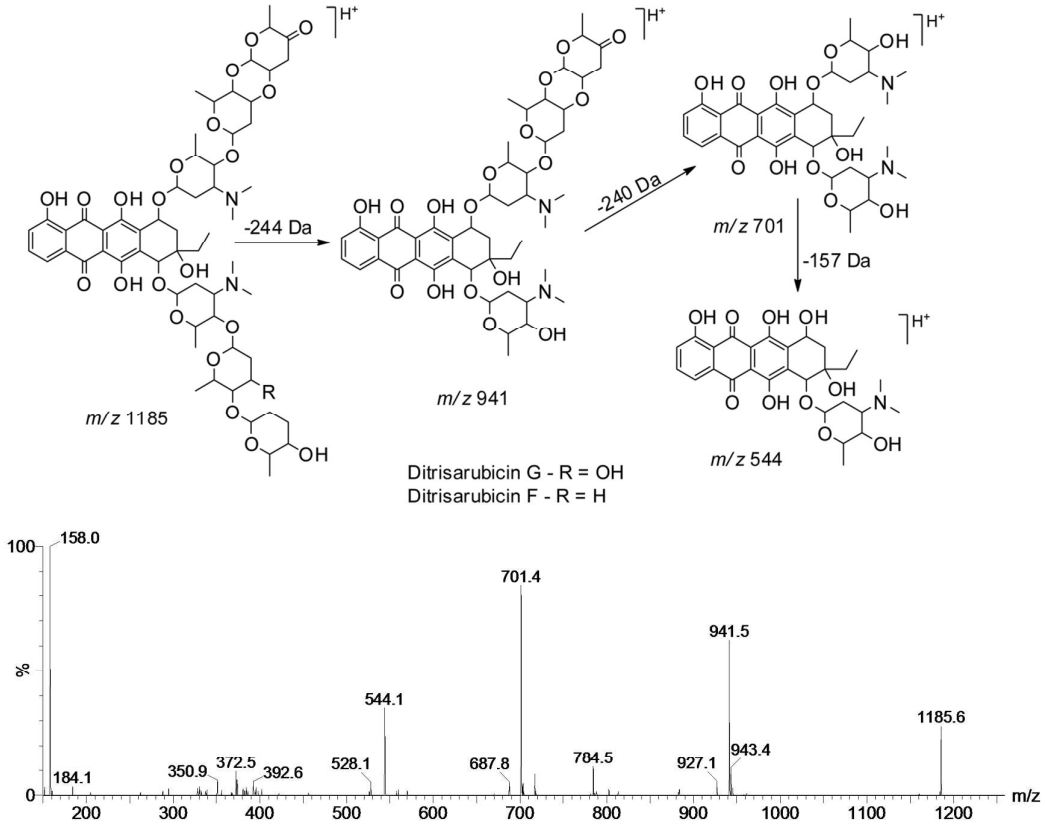
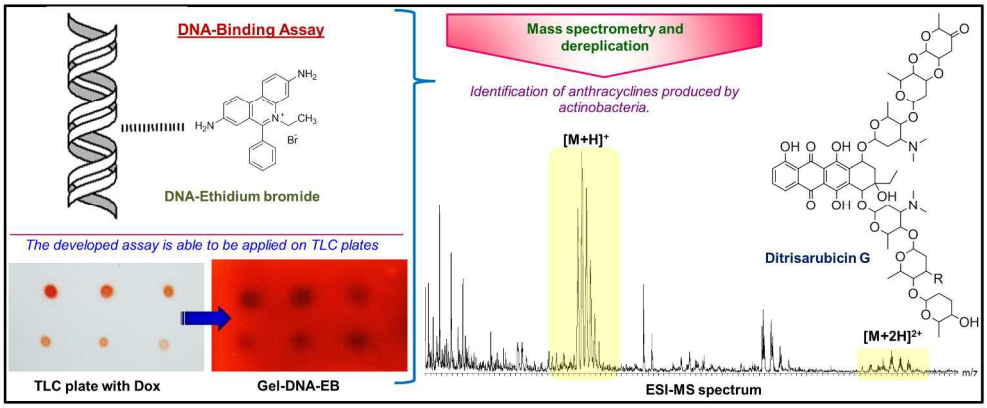


Fig. 4.





226x94mm (300 x 300 DPI)