T. thermophilus Rhamnolipids Induce Cytogenetic Damage on Human Lymphocytes and Bind DNA *in vitro*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AP and TL designed the study, performed the statistical analysis, wrote the protocol and the first draft of the manuscript. Author AP managed the analyses of the study. Authors EA, AM and OS performed the experiments and managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aims: Bacteria including *Pseudomonas* and *T. thermophilus* secretes rhamnose–containing glycolipid biosurfactants called rhamnolipids (RLs), known as bacterial virulence factors. The aim of this investigation was the evaluation of DNA damage induced on human lymphocytes by both RLs itself, secreted in a host organism by pathogens during a bacterial attack or symbiosis and in combination with the camptothecin (CPT), and on calf thymus DNA.
Study Design: Human lymphocytes and calf thymus DNA were treated with isolated *T. thermophilus* RLs for studying DNA damage *in vitro*.
Methodology: RLs DNA damaging action was evaluated by the Sister Chromatid Exchanges (SCEs) methodology, a method for estimating genotoxicity of human exposure to different

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chemicals or other mutagenic agents and by DNA electrophoretic mobility experiments.

Results: RLs at concentrations of 100 and 150 μg/mL reveal significant toxicity. The highest concentration of 200 μg/mL reveals higher genotoxicity. The frequency of SCEs/cell was increased two times over the control level. When CPT, an antineoplastic drug with DNA damaging action, was tested together with RLs the genotoxic activity was reduced significantly ($P<0.01$) compared to the action caused by CPT itself. Sequential increase in the concentration of RLs results in the proportional reduction of Proliferation Rate Index (PRI) which is a cytostatic index. Also, Mitotic Index (MI), a cytotoxic index, was also significantly decreased at concentration of 200 μg/mL RLs. Addition of RLs in the same concentration together with CPT doesn't affect the MI so much. Moreover, RLs are obviously capable for strong binding to plasmid or calf thymus DNA *in vitro*.

Conclusion: RLs exert genotoxicity, cytotoxicity and cytostaticity in human lymphocytes and play probably a protective role for cells against CPT due to RLs' detergent capability to enrobe CPT and DNA, providing a significant property that might support its possible involvement in DNA horizontal transfer phenomena.

1. INTRODUCTION

Many microorganisms especially pathogens like *Pseudomonas* produce rhamnose–containing glycolipid biosurfactants called rhamnolipids (RLs) [1-3]. They are secreted into the extracellular fluid and act as biosurfactants and virulence factors [2]. The reason why microorganisms produce RLs is described [4]. In response to certain environmental signals, bacteria will differentiate from an independent free-living mode of growth and take up interdependent surface-attached microbial communities that are known as biofilms [5]. RLs are amphipathic molecules, which are composed of a hydrophilic sugar moiety (gluconic part) usually containing one or two rhamnoses, and a hydrophobic lipid moiety (lipidic part) which contains one or two hydroxy-fatty acids, providing them tension active properties [6]. First the thermophilic bacterium *Thermus thermophilus* HB8 was shown to produce RLs too, in large amounts using sunflower seed oil, sodium gluconate or glucose as carbon source [7-8]. Later additionally, bacteria *Thermus* sp., *Thermus aquaticus* and *Meiothermus ruber* were found to produce RLs differing in chain length up to unusually long chains with 24 carbon atoms and unsaturation [9].

There is an extended list of reports concerning the biological impact of RLs, including the hemolytic activity, on various cellular constituents [10-14]. Saponin white ($C_{27}H_{42}O_3$), a relative compound of RLs isolated from plants, exhibited hemolytic activity in red blood cells [15], and therefore it has been used as a positive control.

Plethora of biological activities is ascribed to RLs detergent-like properties, and is referred mainly for *P. aeruginosa* biosurfactants that cause changes in the morphology of the plasmatic membrane [16]. Specifically, RLs caused a complete loss in cellular fatty acid content due to release of LPS (lipopolysaccharides) from the outer membrane and that is the probable mechanism of enhancement of cell surface hydrophobicity [17]. RLs also increase binding of insoluble substrates by augmenting cell surface's hydrophobicity through displacement of LPS [18]. RLs cause alterations in the morphology of cell surface of the producer's bacteria and they can disrupt white blood cells, namely neutral polymorphonuclear and monocytes [16,19-22].

Moreover, due to the RLs secretion *P. aeruginosa* attacks respiratory epithelia reconstituted with primary human respiratory cells. The mechanism which is implicated in the internalization of RLs within the host cell membrane followed by tight-junction alterations ending to the presumption that the junction-dependent barrier of the respiratory epithelium is selectively altered by RLs [23]. Increased levels of RLs in the bronchial epithelium of patients with cystic fibrosis with established infection of the bacterium *P. aeruginosa* and the aggravation of the clinical state of the patients were correlated [24]. RLs inhibit the function of the epithelial mucociliary and create alteration to the bronchus affecting the ion transport by decreasing the absorption of sodium and single direction chlorine through bronchial epithelium [25]. Di-RLs were applied on clinical trials on the treatment of persistent diseases like psoriasis, lichen ruber planus, neurodermatitis and human

wound healing [26], and of autoimmune diseases [12]. Finally, RLs exhibit low irritancy and even anti-irritating effects, as well as compatibility with human skin [1].

The *in vitro* antitumor activity of RLs produced by the new strains of *Pseudomonas aeruginosa* BN10 and B189 demonstrated inhibition of proliferation of BV-173 pre-B human leukemia cells by induction of apoptotic cell death [27] and of the growth of human breast cancer cell line MCF-7 and the insect cell line C6/36 respectively [28]. However, RLs elicit the same cytotoxic sensitivity without any distinction between cancer and normal cell by reducing surface tension of culture medium due to their detergent properties [29].

Despite the numerous reports in RLs biological activities, few reports exist concerning their interaction in the level of cellular DNA. To assess the direct effects of RLs on the chromosome the SCEs methodology was used. SCEs are a natural phenomenon related to DNA replication and they involve into the exchange of chromosomal parts between homologous loci of the two sister chromatids during the phase of DNA synthesis and before M phase of cell cycle. The SCEs evaluation is a simple, rapid and very sensitive cytogenetic method for detecting chromosome instability, or DNA unrepair produced by different mutagenic agents, carcinogens or antimutagens [30-34]. SCEs methodology is more sensitive method than chromosome aberrations since induced DNA damage can be revealed by the induction of SCE frequencies even at low concentrations of DNA damaging factors. Furthermore, the other two cytogenetic parameters, the PRI and the MI, are also sensitive indices of cytostaticity and cytotoxicity produced by mutagenic and chemotherapeutic agents respectively.

Irinotecan (CPT) has been chosen as a positive control to reveal any underlying chromosomal damage. CPT is a very common anticarcinogen, a semisynthetic analogue of camptothecin-11, which is an alkaloid isolated from the plant *Camptotheca accuminata*. Its action is focused on the inhibition of topoisomerase I, a valuable enzyme involved in DNA replication [35]. This ability turns CPT into an important DNA damaging agent, which produces high levels of SCEs in human chromosomes.

The aim of this investigation was first the quantitative and qualitative evaluation of DNA damage induced on culture of peripheral lymphocytes using the SCE assay by both RLs itself, the secondary metabolites secreted in a host organism generally by pathogenic microorganisms during a bacterial attack, and in combination with the CPT added. For this purpose three cytogenetic parameters were estimated: (a) the SCEs, (b) the proliferation rate index (PRI), and (c) the mitotic index (MI), which is a qualitative and quantitative index of genotoxicity, cytostaticity and cytotoxicity respectively. Secondly, the aim was to estimate the interaction of RLs with DNA directly *in vitro* on calf thymus double-stranded DNA (dsCTDNA) and plasmid DNA (pDNA) by separating the products of RLs's interaction with DNA by agarose gel electrophoresis.

2. MATERIALS AND METHODS

2.1 Bacterial Strain and Growth for RLs Production

T. thermophilus HB8 (DSM 579) was grown in a rich medium (DSMZ-74) culture used as pre-culture. For RLs production, the bacterium was grown in the presence of sodium gluconate (1.5% w/v) as carbon source at 75°C for 70 h [8].

2.2 Extraction and Analysis of RLs

Produced RLs were extracted from the cell-free supernatant of *T. thermophilus* culture grown in the presence of sodium gluconate [7,8]. Quantification of RLs was obtained by the colorimetric orcinol method [36]. RLs concentration was calculated as described [37].

2.3 Materials and Cell Culture

2.3.1 *In vitro* SCE assay

Human peripheral blood samples were obtained from two male and two female donors, who were healthy medical students, not taking any medication, non-smokers and non-consumers of alcohol. Informed consent was taken from all donors and this study was approved by the University Ethics Committee.

Human peripheral lymphocyte cultures were set up by adding 11 drops of heparinized whole blood from each of the four normal subjects to 5 mL of chromosome medium 1A (RPMI 1640, Biochrom, Berlin). For SCEs evaluation, we add 5-bromodeoxyuridine (BrdU) in a concentration of 5 mg/ml, RLs, CPT and saponin (SAP) at the

beginning of 72 h culture. All cultures were kept in the dark to minimize photolysis of the BrdU and were incubated for 72 h at 37℃. Two hours before harvesting, colchicine was added at 0.3 mg/mL. Metaphases were collected and air-dried preparations were stained by the Fluorescence Plus Giemsa (FPG) technique [38-39] and scored for cells undergoing first mitosis (where both chromatids are dark stained), second (where one chromatid of each chromosome is dark stained) and third and/or subsequent mitosis (where a proportion of chromosomes have both chromatids light stained). Mean SCEs were measured only in suitable second division metaphases and at least 30-40 well spread and differentiated metaphases were blindly counted per culture, because, only at this stage, we were able to observe and count them. In order to establish PRI, at least 200 cells were counted and the following formula was used: PRI = $(M_1+2M_2+3M_{3+})/N$, were M_1 is the percentage of cells at first division, M_2 is the percentage of cells at second division and M_{3+} is the percentage of cells at third and subsequent divisions, while N is the total number of cells counted. Finally for the MIs, at least 2000 activated lymphocytes were determined for each culture [40-41].

2.3.2 Statistical analysis

One-way analysis of variance (ANOVA) and subsequent the Duncan test were performed for all pair-wise comparisons after logarithmic transformation of SCE values. Chi-squared test was used for PRI and MI comparisons and a p-value less than 0.05 was considered to indicate statistical significance.

2.3.3 Materials for DNA electrophoresis

Agarose was purchased from BRL. Tryptone and yeast extract were purchased from Oxoid (Unipath Ltd., Hampshire, UK). All other chemicals were obtained from Sigma. Nucleic acids: Native DNA (dsDNA) type I, highly polymerized from calf thymus gland was purchased from Sigma (D-1501). The DNA stock solution (1 mg/mL) was prepared at 0–4℃ by dissolving the commercially purchased calf thymus DNA in buffer A [50 mM Tris [(hydroxymethyl)aminomethane)–HCl buffer (pH 7.5)] as solvent. Plasmid DNA, pDNA (pET29c) was isolated from *Escherichia coli* (Top 10) using the GenEluteTM HP endotoxin-free plasmid maxiprep preparation (Sigma-Aldrich), according to the manufacturer's specifications. The intercalative dye ethidium bromide (EthBr), were purchased from Sigma. Stock solutions of RLs

were prepared at a final concentration of 500 µg/mL by dissolving RLs in water.

2.3.4 RLs interaction with dsCT-DNA or pDNA

The binding and/or cleavage reaction of dsCT-DNA or pDNA exposed to RLs was monitored by DNA mobility shift experiments in agarose gel electrophoresis. Generally, the efficiency of the DNA interaction with a compound reflects to the electrophoretic mobility and it is dependent from the concentration of the compound as well as the form and structure of the DNA substrate used. When a DNA band after interaction with a compound displays a retardation in its electrophoretic mobility compared to control, this effect could be attributed to the binding of certain molecules of the compound on DNA molecules able to increase its molecular weight. While when in electrophoretic mobility a precession of the DNA band was observed after the pre-referred interaction this fact could be attributed to the damage of the initial DNA substrate mirrored to a decrease of its molecular weight. DNA molecules with lower molecular weight from that of the initial DNA molecule migrate faster, while DNA molecules with higher molecular weight delay compared with the mobility of the initial DNA band. Reactions contained aliquots of an amount (µg) of nucleic acid (CT-DNA DNA or pDNA) as indicated in the legends, which were incubated at 37℃ for 30 min in the presence of various concentrations of RLs in a buffer A to a final volume of 20 µL. Reaction were terminated and separated in agarose gel electrophoresis as previously described [39].

3. RESULTS

3.1 Genotoxicity, Cytostaticity and Cytotoxicity of RLs on Human Lymphocytes

For the evaluation of genotoxicity, cytostaticity and cytotoxicity caused by RLs on human lymphocytes the following three parameters have been evaluated: SCEs, PRIs and MIs, respectively for every experimental procedure that has been completed three times. Every one of these consisted of culture: control, with CPT, with RLs in concentrations of 100, 150 and 200 µg/mL, with their combinations with CPT and finally with SAP, as positive controls, in concentrations of 1 and 2.5 µg/mL.

First, as it is shown in Fig. 1 and Table 1, RLs in concentration of 100, 150 and 200 µg/mL produced a statistically significant induction of

SCEs compared to controls that indicate the possible genotoxic effects of RLs in these concentrations. In parallel CPT tested alone produced statistically significant induction of SCEs compared to controls. However, when RLs were tested in different concentrations supplemented with CPT SCE frequencies display statistically significant increase compared to both control and RLs cultures, but there wasn't found any difference between the combinations of CPT plus RLs. The above double combinations (CPT plus RLs) compared to CPT alone, showed statistically significant (P<0.01) reduction in SCEs. This observation can be interpreted as a protective action of RLs against CPT. Furthermore, adding SAP as positive control, in concentrations used (1 and 2.5 µg/mL), it didn't cause significant change in SCEs in these concentration compared to control and between the two concentrations. Similar results showed that RLs biosurfactants decrease the toxicity of chlorinated phenols against *Pseudomonas putida* DOT-T1E [42]. Secondly, as for PRIs, the addition of CPT produced a strong delay (P<0.01) in cell proliferation rate (PRI) compared to control. However, the increase of RLs concentrations showed a very strong significant decrease of PRIs (P<0.01). Similarly, a decrease (P<0.01) of PRI was observed from the double combination of CPT plus RLs of 200 µg/mL compared to controls (Fig. 2).

At the end, the qualitative x^2–test showed a decrease of MIs in cultures with RLs compared to control and specially a very strong decrease (P<0.01) in concentration of 200 µg/mL of RLs (Fig. 3). The above results are also collectively shown in Table 1.

3.2 RLs interaction with CT-DNA by DNA Mobility Shift Experiments in Agarose Gel Electrophoresis

Extracellular nucleic acids among other polymeric substances (EPS) alter the surface properties of the bacteria themselves to either promote or prevent initial attachment to a surface [43-44], and extracellular DNA has been shown to be one of the principal factors involved in biofilm formation for *P. aeruginosa* while treatment with DNase I inhibit it [45]. In parallel, RLs may be able to maintain open channels by affecting cell-cell interactions and the attachment of bacterial cells to surfaces and their production affects biofilm architecture in *P. aeruginosa* PAO1 [5]. Thus, the concept was to investigate a possible interaction between RLs and DNA *in vitro*. This interaction was studied first by treating calf thymus ds DNA with two amounts of RLs. When calf thymus ds DNA was incubated with RLs under investigation an up-shift of a small amount of the DNA band was observed to move, resulting in the formation of a new band in the top of the gel, while the main DNA band appeared more wide, diffused and degraded because of the detergent properties of RLs (Fig. 4). This suggestion mirrored binding or coating of DNA with RLs.

Table 1. Effects of RLs on human lymphocytes

Treatment	SCEs/Metaphase ± SEM (range)	Proliferation rate index (PRI)	Mitotic index (MI)
1. Control	3,70±0,29(1-8)	2,28	26,50
2. CPT 0.5 µg/mL	31,75±1,14(17-40)	2,18	23,33
3. RLs 100 µg/mL	5,34±0,44(2-11)	2,27	24,75
4. RLs 150 µg/mL	6,00±0,88(1-12)	2,17	23,00
5. RLs 200 µg/mL	7,56±0,34(2-18)	1,70	13,75
6. CPT 0.5 µg/mL + RLs 100 µg/mL	19,53±0,97(11-25)	2,28	25,00
7. CPT 0.5 µg/mL+ RLs 150 µg/mL	19,90±1,33(10-27)	2,33	24,50
8. CPT 0.5 µg/mL + RLs 200 µg/mL	22,23±1,13(9-31)	2,19	23,50
9. Saponin 1 µg/mL	4,06±0,39(1-8)	2,23	25,00
10. Saponin 2.5 µg/mL	4,42±0,33(1-9)	2,18	22,00

For SCEs, using ANOVA test, the F value was 134,995 (P<0.05 between cultures) and comparing with Tukey-test, it was found P<0.01 for 1/2, 1/3, 1/4, 1/5, 1/6, 1/7, 1/8, 2/6, 3/6, 4/7, 5/8 and P<0.05 for 2/7 and 2/8 comparisons. For PRIs, using x^2–test, it was found P<0.01 for 1/2, 1/4, 1/5, 1/10, 2/6, 2/7, 3/4, 3/5, 3/6, 3/10, 4/5, 4/7, 5/8, 5/9, 5/10, 6/8, 7/8. For MIs, using x^2–test, it was found P<0.01 for 1/5, 2/5, 3/5, 4/5, 5/8, 5/9 and 5/10 comparisons
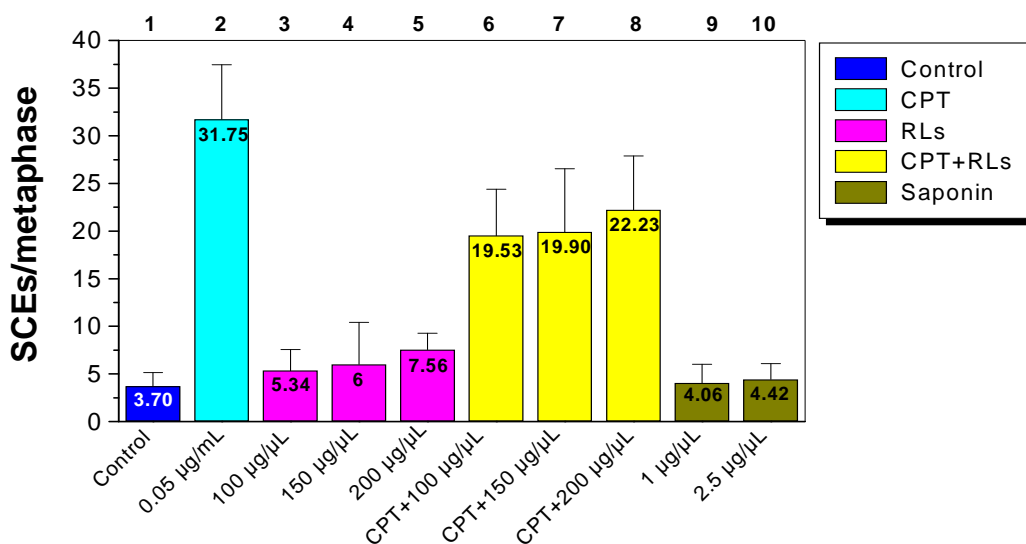
Fig. 1. The change in mean number of chromatid exchanges per metaphase for cell cultures exposed to CPT at final concentration of 0.5 µg/mL, RLs at concentrations of 100, 150 and 200 µg/mL, CPT and the corresponding concentration of RLs and saponin in concentrations of 1 and 2.5 µg/mL for 72 h. For the observation and assessment of chromatid exchanges was used the method Fluorescence plus Giemsa (FpG) slightly modified (where $P<0.01$ for 1/2, 1/3, 1/4, 1/5, 1/6, 1/7, 1/8, 2/6, 3/6, 4/7, 5/8 and $P<0.05$ for 2/7 and 2/8 comparisons respectively)
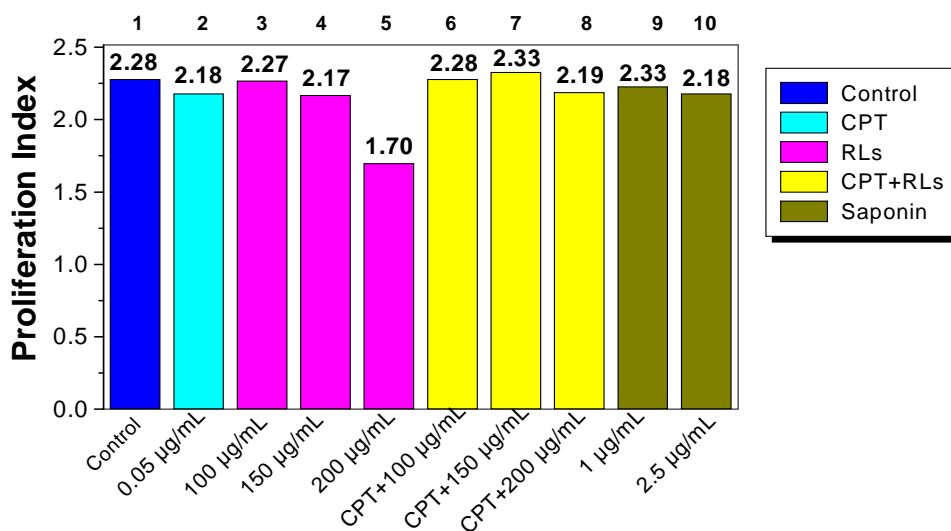

Fig. 2. The change in mean of PRI in lymphocyte cultures exposed to CPT at final concentration of 0.5 µg/mL, RLs at concentrations of 100, 150 and 200 µg/mL, CPT and RLs at the corresponding concentrations and saponin at concentrations of 1 and 2.5 µg/mL for 72 h. The proliferation rate index was based on the determination of the proportion of metaphases 1st, 2nd or 3rd+ generation (where $P<0.01$ for 1/2, 1/4, 1/5, 1/10, 2/6, 2/7, 3/4, 3/5, 3/6, 3/10, 4/5, 4/7, 5/8, 5/9, 5/10, 6/8, 7/8 comparisons)
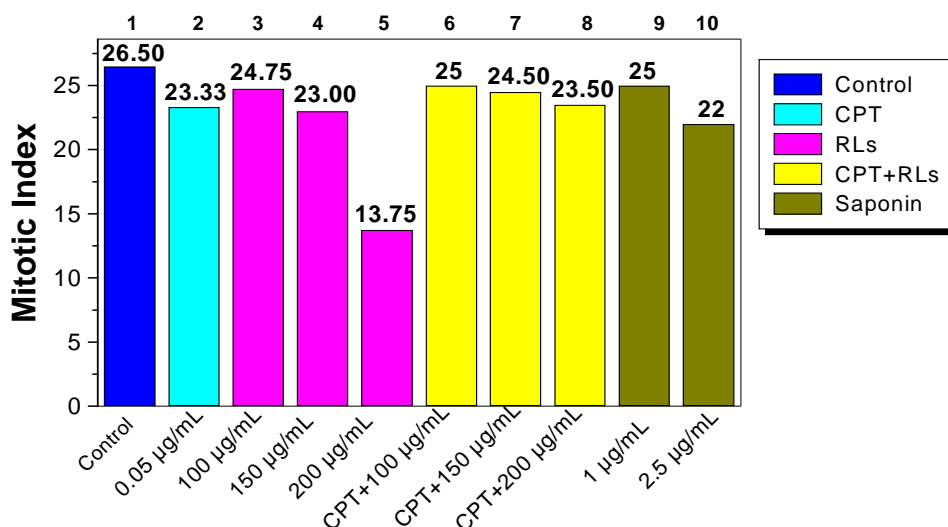
Fig. 3. The change in mean rate of MI in cell cultures exposed to CPT at final concentration of 0.5 µg/mL, RLs at concentrations of 100, 150 and 200 µg/mL, CPT and RLs at the corresponding concentrations and saponin at concentrations of 1 and 2.5 µg/mL for 72 h. Measurement of MI requires the measurement of metaphases encountered by a representative set of nuclei, that is, not undergoing mitosis (where P<0.01 for 1/5, 2/5, 3/5, 4/5, 5/8, 5/9 and 5/10 comparisons)
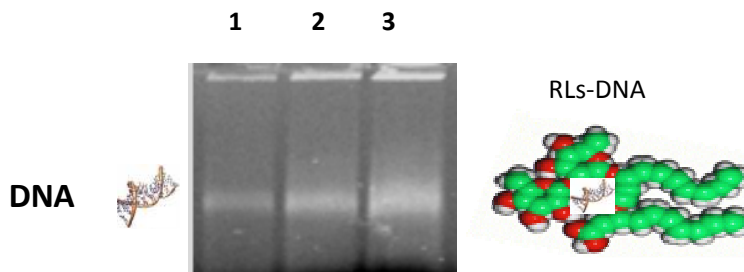

Fig. 4. Agarose (1% w/v) gel electrophoretic pattern of calf thymus DNA after 2 h of electrophoresis duration. Each sample containing 3 µg of calf thymus DNA that was treated with the indicated amount of RLs at 37 °C for 60 mi n. Lane 1: control, calf thymus DNA incubated without treatment in water; Lanes 2 and 3: calf thymus DNA treated with 16. 66 and 33.33 µg of RLs, respectively

3.3 Interaction of RLs with pDNA

To take onto account the secondary and supercoiled structure of the pDNA, the possible direct interaction of RLs on the pDNA was also studied by treating pDNA with four different amounts of RLs and then by separating the products in agarose gel electrophoresis. When pDNA was incubated with RLs, an up-shift of the two pDNA bands (supercoiled and relaxed) was observed resulting in the formation of new bands with delayed electrophoretic mobility. Additionally a new band in the top of the gel near the well that increased quantitatively with the amount of RLs added, with a concomitant disappearance of the two pDNA bands. These results mirrored the binding of RLs with pDNA and formation of RLs-pDNA complexes with delayed electrophoretic mobility attributed to the bio-surfactants nature and properties of RLs like detergents that may coat pDNA (Fig. 5). This finding might be proved significant whether RLs implicated and mediate DNA transfer with this manner between bacteria.

4. DISCUSSION

Despite the numerous reports in RLs biological impacts in cellular constituent's, rare reports exist concerning their interaction in the level of cellular
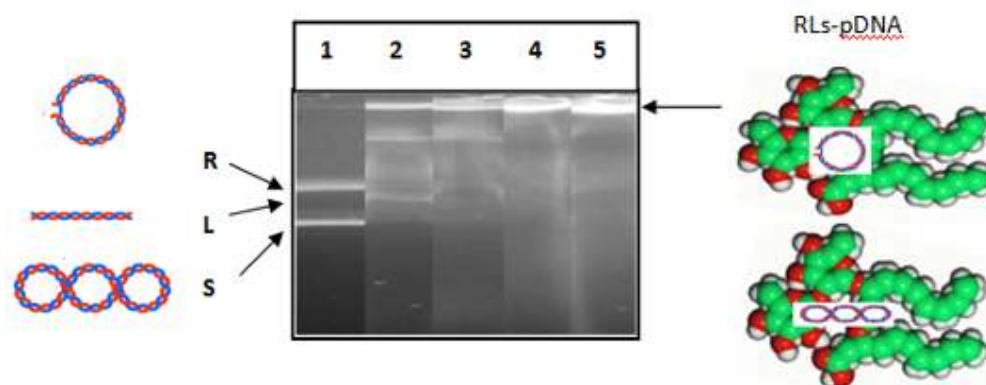
Fig. 5. Agarose (1% w/v) gel electrophoretic pattern of an EthBr stained mixture of supercoiled and relaxed DNA, plasmid DNA (pET29c) after 2 h of electrophoresis duration.
Each sample containing 3 µg of plasmid DNA that was treated with the indicated amount of RLs at 37℃ for 60 min. Lane 1: control, plasmid DN A incubated without treatment in water (zone S represents the supercoiled form of plasmid (supercoiled), zone L represents the linear form of the plasmid (linear) and zone R represents the relaxed form of the plasmid (relaxed).; Lanes 2, 3, 4 and 5: plasmid DNA treated with 6.66, 16.66, 33.33 µg and 56.66 µg RLs, respectively

DNA. Lymphocytes displayed chromosomal fragility when are exposed to RLs, secreted by pathogens microorganisms during provisional or more permanent symbiosis with humans. RLs secretion by bacteria is favored under nutrient limitation especially phosphate [8], and this is crucial especially in humans when the host organism is found mainly under specific conditions of homeostatic imbalance like nutrients limitation of e.g. phosphate (in hypophosphatemia), nitrogen etc. RLs induce cytotoxicity as it was assessed by MTT test, and pronounced alterations in morphology on human skin fibroblasts, when they were co-cultivated with two different kinds of purified RLs, originated from *T. thermophilus* cultures grown in different carbon sources previously elucidated by our group [46].

RLs reported to inhibit DNA synthesis in A431 human epidermal cells but did not caused chromosomal aberrations [12], and to interact even with DNA transcriptional and translational machinery [26] after internalization via a cell-receptor, passage through the phospholipid bilayer of the cell membrane and the probable formation of complexes with serum constituents. In contrast it was also reported that the ability of RLs (II) to cause SCEs on CHO cells, showed that SCEs method did not show significant differences between control and treated cultured cells [12]. Additionally, structural chromosomal aberration analysis was performed for evaluation

of RLs impact on DNA integrity with RLs (II) [12], showed that the low concentration of 31.25 µg/mL caused a large number of structural chromosomal aberrations when compared to the untreated control cells. The middle concentration of 62.50 µg/mL in the same incubation time decreased the tendency of chromosomal aberrations, while for the largest concentrations tested the values were almost the same as in the control group. However, a significantly different behaviour was observed, when RLs were tested for chromosomal aberrations after 16 h incubation time. Generally structural chromosome damages increased several fold, as well as the number of cells with 2 to 4 aberrations. Thus, the authors declare notably that the number of aberrations decreased as the concentration increased [12].

Additionally, for comparison, it is posed near the effect of *P. aeruginosa* di-RLs on fibroblasts which were recorded by others also as a cytotoxic effect as follows: Di-RLs at 50–200 µg/ml, significantly inhibited proliferation of fibroblasts while di-RLs at higher concentrations (0.5 and 1 mg/ml), showed a cytotoxic effect on fibroblasts, affecting cell membranes that were damaged and the cytoplasmic structures appeared to disintegrate, demonstrating a cell necrosis process [47]. Moreover, chromatin condensation and margination, nuclear fragmentation, plasma membrane blebbing, and presence of apoptotic bodies were reported in

RLs-treated tumor cell cultures [48,49]. Saponin tested in SCEs causes cytogenetic damages in cultured human lymphocytes. Saponins possess detergent-like properties and increase the permeability of cell membranes without destroying them [50]. It has been recently pointed out that the triterpenoid saponin avicin can induce apoptosis through the formation of channels within the cell membrane [51]. All these results are in agreement with our findings.

Moreover, it was also demonstrated that RLs are able to bind or mask DNA due to their detergent properties, providing significance on their possible physiological role in different processes. A high-molecular-weight bioemulsifier was previously reported that coat the bacterial surface and can be transferred horizontally to other bacteria, thereby changing their surface properties and interaction with the environment. This horizontal transfer of bioemulsifiers from one bacterial species to another has significant implication in natural microbial communities, co-aggregation and biofilms [52].

Moreover other authors support the hypothesis that RLs facilitate the transport of flagellin, a bacterial virulence factor, across the *stratum corneum,* and this RLs-based shuttle system is not limited only to flagellin [53], but could constitute a universal transport system throughout the skin barrier [53], shuttling even host derived factors like the cytokines by the participation of this RL-based delivery system. RLs vesicle may potentially be able to fuse with the cell membrane and release its contents into cytoplasm like chemo-therapeutics agents etc [53].

The experimental results demonstrated also that CPT likely encased by RLs, was shuttled easier to the cells and consequently causes a reduced number of SCEs, resulting from its reduced capacity as anticarcinogen due to its coating by RLs. Thus, it can be assumed that other compounds like mutagens or other factors on cells including DNA might be delivered finally resulting in activation or suppression of a specific host response such as SCEs or CPT capacity. The DNA coating capacity prompted us to the fair question, why to exclude the possibility of transport of agents or other bacterial components into the eukaryotic cells including DNA via a RLs-dependent manner, in case that bacteria are in favorable environment in host cells. Moreover, *P. aeruginosa* is capable of utilizing as a nutrient source extracellular DNA that is ubiquitous in various environments and is a rich source of carbon, nitrogen and phosphate [54]. The result of the direct binding of RLs to DNA might support this hypothesis.

5. CONCLUSION

The addition of RLs in culture of human lymphocytes influences the three cytogenetic indices (SCEs, PRIs and MIs) producing genotoxic, cytostatic and cytotoxic effects respectively. It is obvious that their presence in lymphocyte cultures decreases the genotoxic effects of CPT probably due to detergent-like activity of RLs that might coat the molecules of CPT and thereafter at one hand improve the uptake of CPT, but at the other hand prevent the CPT action due to its encasement into the RLs layer. Thus, we believe that RLs worked as scavengers of CPT molecules and so they reduced CPT's activity. On the other hand RLs bind to pDNA, and this indicates that they have the ability to be involved in DNA horizontal transfer phenomena.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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