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Production of Anticancer Compound from Marine *Lactobacillus rhamnosus*

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Abstract: Marine resources possess unique compounds able to be specific to target and reduce the risk of unwanted side effects of the traditional chemotherapy. In this study, a marine antitumor producer was isolated and identified as *Lactobacillus rhamnosus* using the 16s rRNA gene sequence. Optimization for antitumor production was carried out using Plackett-Burman design, leading to increasing the activity to 93% compared with the basal conditions using the Red Potato Disk Bioassay. *In vivo* cytotoxicity assay for the *L. rhamnosus* ethyl acetate extract showed similar inhibition% (65.4%) in comparison to Doxorubicin, While *in vitro* 3-[4,5-dimethylthiazole-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay the extract showed unexpected behavior. The chemical characterization indicate the extraction of 1,2-cyclohexanedicarboxylic acid, cyclohexylmethyl nonyl ester using Gas Chromatography / Mass Spectrometry (GC-MS).

Key words: Alternative · Natural · Marine · Anticancer · Doxorubicin

INTRODUCTION

The global incidence of cancer raised to an estimated 14 million new cases in 2012 and this is expected to rise to an annual 19.3 million cases by 2025 [1]. Novel drugs are still a priority goal for cancer therapy to avoid their undesirable side effects which include; nausea, vomiting, hair loss (alopecia), early menopause, weight gain, obstipation, diarrhea, memory problems,..etc. [2, 3]. Marine environment has proven to be a rich source for new natural products and chemicals. They can be applied in pharmaceutical, food and cosmetic industries in addition to the production of nutraceuticals [4, 5].

The present study was a trial to avoid the side effects of the most commonly-used anticancer chemotherapeutic drug Doxorubicin through a discovery of an alternative antitumor agent produced by a marine *Lactobacillus rhamnosus* which was optimized and chemically characterized using the GC-MS spectrometry.

MATERIALS AND METHODS

Collection of Samples: Sediment, seawater and some algal samples of *Ulva fasciata* and *Corallina mediterranea* were collected from different coastal area in Alexandria, Ras El-Bar, Marsa Matrouh and Sharm El-Sheikh, Egypt [6].

Isolation of Different Marine Bacteria : The isolation was carried out using marine nutrient agar plates (Sigma com.). Plates were incubated at 30°C for 1-2 days [6] and the algal samples were used according to Singh *et al.* [7]. The obtained colonies were kept at 4°C for further investigations.

Selection of Anti-tumor Producers: Red Potato Disc (RPD) Bioassay was used to select antitumor producers using a bacterium causes the crown gall disease *Agrobacterium tumefaciens* [8, 9]. Potato discs were examined separately compared to the blank potato discs (B) applied with 50 µl/disc sterile sea water, the first control potato discs (C_1) was applied with 50 µl/disc *A. tumefaciens* suspension and the second control potato discs (C_2) was applied with 50 µl/disc pre-treated *A. tumefaciens* with 100 µl Ampicillin (1000 mg/5 ml) to eliminate the infection effect of variable *A. tumefaciens* cells on the potato discs without affecting its Ti plasmid activity. The plates were incubated at room temperature for 12-14 days then all discs were stained with Lugol's Reagent [10].

Bio-Toxicity Test Using Artemia Salina as a Biomarker: The biotoxicity of different concentrations 250, 500, 750 and 1000 ppm [v/v] of the tested supernatant of the bacterial suspension was detected according to Meyer

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Table 1: Factors affecting the production of antitumor agents using Plackett-Burman experiment.

		Tested Level		
Factors	Symbol	-1	0	+1
Peptone (g/l)	Pept.	5	10	20
Beef extract (g/l)	BE	5	10	20
Sodium chloride (g/l)	NaCl	2.5	5	7.5
Temperature (°C)	Temp.	25	30	37
pH	PH	6	7	8
Inoculum size (ml)	IS	1	2	4
Incubation time (day)	IT	2	3	4

et al. [11] using 24 h old *Artemia salina* nauplii. The mortality percentages and the half-lethal dose (LD_{50}) were determined after 24-48h according to Niaz *et al.* [12].

Molecular Identification Process: The most potent antitumor producer was chosen for identification using 16s rRNA gene sequence. DNA was extracted using GeneJET Genomic DNA purification kit (Thermo). The preparations were analyzed on a 0.7% agarose gel and then determined spectrophotomertically. The PCR-amplification was carreied out using reommended primers; F (3' AGA GTT TGA TCC TGG CTC AG-5') and R (3'- GGT TAC CTT GTT ACG ACT T -5'). The GeneJET™ purification column was used for cleaning up the PCR product. The used nucleotide sequence of the 16s rRNA gene was compared with the data available in the GenBank.

Application of Plackett-burman Design: The marine nutrient broth was selected and optimized using Plackett-Burman experimental design [13, 14]. Seven independent variables were screened in nine combinations organized according to a design matrix compared to the basal conditions; the symbols and the applied level of these variables were presented in Table (1).

Scaling up and Extraction Process: Five ml *L. rhamnosus* suspension was prepared and used to inoculate 1000 ml optimized marine nutrient broth. Then the extraction of bioactive metabolites was made using an organic solvent ethyl acetate (1:1 v/v) for 24 h [15].

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis: The components of the most potent bacterial extract were analyzed using an Agilent GC-Ms (7890A GC and 5975C inert MSD) equipped with a splitless injector and a capillary DB-5 Ms column [30m × 0.25mm × 0.25im (Agilent p/n 122-5532UI)]. The chemical components were identified by comparison of their mass fragmentation patterns to the reference standard data [16].

***In vitro* Cytotoxicity Assay:** The produced antitumor extract was tested to inhibit two immortalized cell types; the colon cell line (HCT116) and the liver cell line (HepG2). The 3-[4,5-dimethylthiazole-2-yl]-2,5 diphenyltetrazolium bromide (MTT) was used for this cytotoxicity assay [17].

***In vivo* Cytotoxicity Assay:** This experiment was carried out according to Abdel-Aziz *et al.* [18], it was divided into five groups, each group composed of six female Swiss Albino mice as follow: Group 1: six mice were considered as negative control without injection with Ehrlich Ascites Carcinoma (EAC),Group 2: six mice were considered as positive control injected with 10^6/ml EAC only without treatment, Group 3: six infected mice were treated intravenous every week with the tested anti-tumor agent at 0.5 mg/kg dose level for three weeks, Group 4: six infected mice were treated intravenous every week with the tested anti-tumor agent at 1 mg/kg dose level for three weeks and Group 5: six infected mice were treated intravenous every week with standard chemotherapeutic drug (Doxorubicin) at the recommended dose level (2.5 mg/kg) for also three weeks. During the three weeks of injection the whole body weight and the tumor volume (TV) were determined weekly using a digital vernier caliper. Tumor volume (TV) of each animal was calculated using the following formula:

$$TV = \text{length (mm)} \times [\text{width(mm)}]^2 \times 0.52$$

Tumor growth inhibition (TGI) was calculated as follows:

$$TGI (\%) = 1 - (\text{RTV of the treated group at the day of measurement})/(\text{RTV of control group at the day of measurement}) \times 100.$$

where RTV = Tumor volume at the day of measurement / Tumor volume at the initial day.

RESULTS

Screening for Antitumor Producers: Seventy nine bacterial strains were isolated from the collected seawater, sediment and algal suspension samples. On using the Red Potato Disc Bioassay it was observed only seven microbial isolates MT5, RBW1, MMS7, MA3, GW11, MF2 and RBS4 showed inhibition of the Ti plasmid of

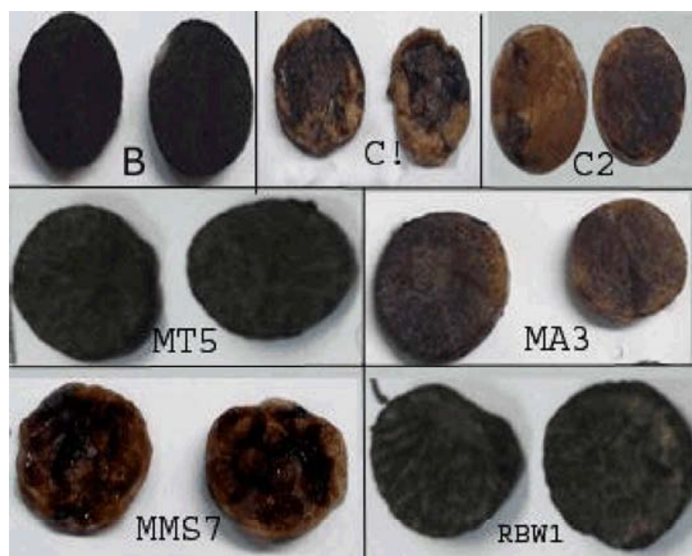

Fig. 1: Photographs of the potato discs show the antitumor activity of the marine bacterial isolates (MT5, MA3, MMS7 and RBW1) compared to; B (Potato discs + sterile seawater), C1(Potato discs + *A. tumefaciens*) and C2(Potato discs + *A. tumefaciens* + Ampicillin) after 15 days of incubation.

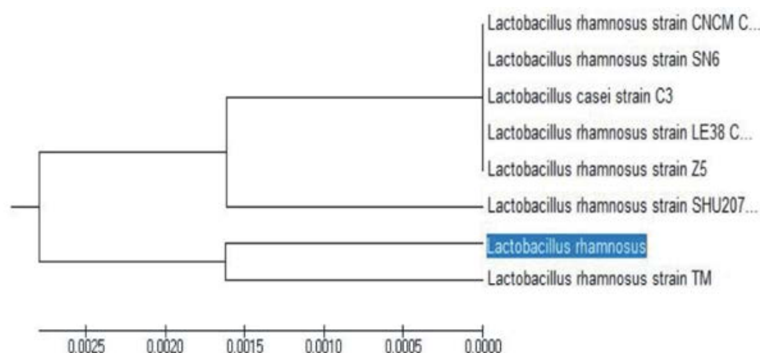

Fig. 2: The phylogenetic relationships between the the marine antitumor producer *Lactobacillus rhamnosus* and other 16s rRNA sequences of the most closely related bacteria species.

A. tumefaciens. But after two successive experiments it was found the most antitumor producers were MT5, RBW1, MMS7 and MA3 isolates. The inhibition % was 87.5, 85, 80 and 77.5, respectively (Fig. 1).

Bio-Toxicity Assay: The LD_{50} of the supernatants of the tested isolates MT5, MMS7, MA3 and RBW1 were 831,750, 645 and 575ppm, respectively, using *A. salina* as a biomarker.

Genotyping Identification of the Most Potent and Safe Antitumor Producer: According to the results obtained from the Red Potato Disc Bioassay and the biotocicity test the most promising isolate was MT5, it was identified as *Lactobacillus rhamnosus* with identity of 99% compared to several strains of *Lactobacillus rhamnosus* presented in the GenBank database especially *Lactobacillus rhamnosus* strain TM, (Fig. 2).

Optimization of L. Rhamnosus Growth and Antitumor Activity Using Plackett-Burman Experimental Design: The growth of *L. rhamnosus* using the marine nutrient broth as a basal culture medium showed an optical density (OD) of 0.583 after 90h of incubation with an antitumor activity of 87%. The obtained main effect using Plackett-Burman experimental results (Fig. 3), as well as the t-test values indicated the optimum conditions for maximum antitumor activity (93%) were as follow; peptone, beef extract, sodium chloride, pH value and inoculum size must be adjust at their high levels (+1);

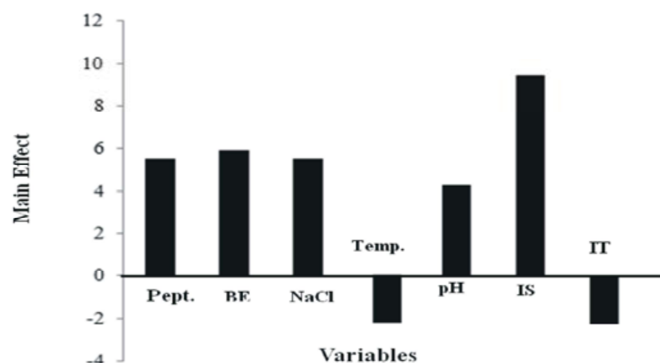

Fig. 3: Elucidation of the main effect of the tested culture factors affecting the production of antitumor agent by *L. rhamnosus* using the Placket-Burman design.

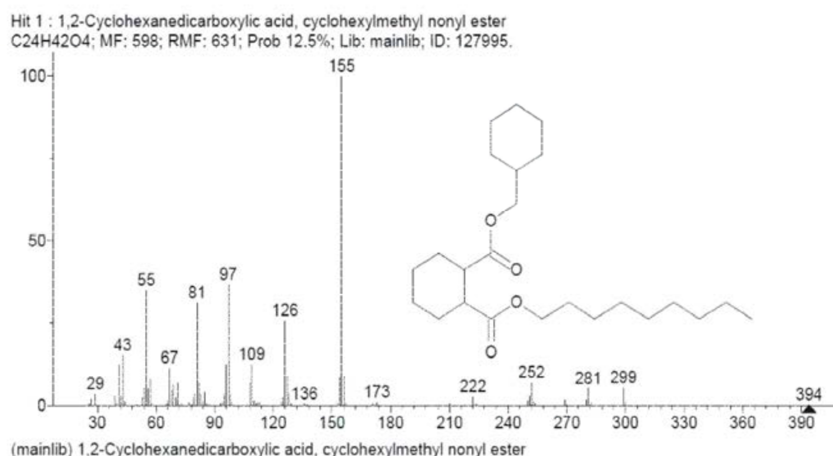

Fig. 4: Mass spectrum of 1,2-Cyclohexanedicarboxylic acid, cyclohexylmethyl nonyl ester

Table 2: The IC_{50} values of CDCMNE (iM) against liver cell line (HepG2) and Colon cell line (HCT116) compared to Doxorubicin (DOX).

Tested product	Liver cell line (HepG2)	Colon cell line (HCT116)
DOX	1.9±0.498	1.3±0.791
CDCMNE	45.7±1.930	47.2±0.929

Table 3: Anti-tumor activities of CDCMNE and Doxorubicin against EAC solid tumor model.

Experiment Time (day)	The studied groups (n=24) Mean of tumor volume (TV) (mm^3) ±SD				*F value
	Control (n=6)	CDCMNE $Dose_1$ (n=6)	CDCMNE $Dose_2$ (n=6)	DOX (n=6)	
0 Day	233±6.6	236±9.0	232±8.5	227±14.2	20.22
7th day	822±6.8	657±13.1	513±14.1	409±16.1	1146.38
14th day	1771±17.8	1215±25.0	901±21.8	687±13.5	3327.01
21th day	3053±22.3	1689±21.5	1206±28.7	1029±17.4	9674.00
*F value	40288.55	7447.173	2689.276	3108.470	

*Significant at level P<0.05, control: Infected and untreated female Swiss Albino mice with the anti-tumor agent. CDCMNE $Dose_1$: 0.5 mg/kg body weight, CDCMNE $Dose_2$: 1.0 mg/kg. DOX: Doxorubicin dose 10mg/Kg body weight.

(20 g/l, 20 g/l, 7.5 g/l, 8 and 4 ml of $4.5x10^8$ cells/ml, respectively) while the temperature and incubation time must be adjusted at their low levels (-1); (25°C and 2 days, respectively).

Gas Chromatography–Mass Spectrometry Analysis: The ethyl acetate extract of *L. rhamnosus* was subjected to GC-MS, one compound namely 1,2-Cyclohexanedicarboxylic acid, cyclohexylmethyl nonyl

ester (CDCMNE) was found to be the major component in this extract with almost 100% peak area and retention time of 35.136 min. The molecular formula was $C_{24}H_{42}O_4$ and the molecular weight of 394.31 g/mol, compared with libraries search (mainlib and replib) Fig. (4).

Cytotoxicity (MTT) Assay: According to the obtained data (Table 2), CDCMNE showed high IC_{50} against liver cell line (HepG2) and the colon cell line (HCT116), respectively, compared to the standard Doxorubicin as a positive control.

***In vivo* Evaluation:** The mean tumor volume (TV) showed significant decrease at $P < 0.05$ for both tested doses of CDCMNE in comparison with the control. After 21 days of the treatment with 0.5 and 1.0 mg/kg CDCMNE doses they showed tumor inhibition% of 45.0 and 61, respectively. While, the tested Doxorubicin dose (2.5 mg/kg) showed 65.4% inhibition compared to the control after the same time of exposure (Table 3).

DISCUSSION

From the obtained data in this study the marine isolate MT5 was selected as potent and safe antitumor producer. It was identified as *Lactobacillus rhamnosus*, it was reported to have anti-obesity, anti-inflammatory properties [19] and anti-hyperglycemic effect [20]. *L. rhamnosus* CCFM1107 showed protective effects on alcoholic liver injury since it reduce the oxidative stress [21]. Tiptiri-Kourpeti *et al.* [22] reported that lactobacilli strains exert immunomodulatory, anti-inflammatory and anti-carcinogenic properties. Also, Boopathy and Kathiresan [23] mentioned that it decreases the induction of experimental colon cancer. Moreover, Faghfoori *et al.* [24] worked on the probiotic properties of lactobacilli.

In addition, the results of using the optimized marine nutrient broth culture medium through the Plackett-Burman design and the Red Potato Disk Bioassay indicated the antitumor activity of *L. rhamnosus* reached to 93% with 6% increase compared to the basal medium. Likely, Devi *et al.* [25] used the marine broth culture medium with high percentage of salt in production and characterization of the active factors of marine bacterial isolates. While, El-Naggar *et al*. [9] and Bizuye *et al*. [26] mentioned that the marine actinomycetes preferred the oatmeal nitrate agar medium as the best culture medium for microbial growth and antitumor activity.

The *in vivo* results of CDCMNE showed to be an alternative promissing anticancer agent acting as the chemotherapeutic drug Doxorubicin. Similarly, several scientists worked on the biological activities of CDCMNE and its derivatives as anticancer drug. It was reported that CDCMNE possess interesting *in vivo* antitumor activity towards L1210 leukemia in mice [27], exhibited selective cytotoxicity against a tumorigenic cell line [28] and two cancer cell lines melanoma [29]. On the other hand, Selvin *et al.* [30] stated that cyclohexanecarboxylic acid, hexyl ester has antimicrobial activity. Kadhim *et al.* [31] mentioned that cyclohexanecarboxylic acid, 2-hydroxy-, ethyl ester has antipyretic and anti-inflammatory activities. However, the produced CDCMNE showed unexpected behavior towards the tested cell lines and this is may be due to its specificity against other untested cell lines or it may show its activity *in vivo* tests only rather *in vitro* examinations. This unexpected mode of action was explained through the results obtained in Table 3 where the extracted CDCMNE showed similar *in vivo* anticancer activity as that of the standard chemotherapeutic drug Doxorubicin even on appling 2/5 of its recommended dose. Likely, Zhu *et al.* [32] stated although bostrycin and its derivatives didn't seem to be active *in vitro* experiments, SZ-685C displayed significant *in vivo* antitumor activity against adriamycin-resistant human breast xenografts.

CONCLUSION

The isolated marine *L. rhamnosus* could produce an alternative promising natural marine antitumor compound, 1,2-Cyclohexanedicarboxylic acid, cyclohexylmethyl nonyl ester. It showed *in vivo* cancer treatment rather *in vitro* experiments compared to Doxorubicin. Synergetic combination between CDCMNE and Doxorubicin is recommended to enhance anticancer activity and/or avoid the bad chemotherapy side effects.

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