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Production, characterization and anticancer activity of *Candida bombicola* sophorolipids by means of solid state fermentation of sunflower oil cake and soybean oil

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SUMMARY: The production of sophorolipids by *Candida bombicola* NRRL Y- 17069 grown in a mixture of sunflower oil cake and crude soybean oil as economic substrates with different fermentation techniques was studied. The highest yield (49.5 g·100 g^{-1} substrates) was obtained from solid state fermentation after employing a new concept for extraction by methanol (E I) followed by ethyl acetate (E II), then partially purified with hexane (E III). The course of time of fermentation was also studied, and E I extracted of the 12th day showed the minimum surface tension (45 mN·m^{-1}) at a critical micelle dilution (CMD) of 10% concentration. The produced sophorolipids were characterized and confirmed by FTIR and ^{1}H NMR spectroscopy. The anticancer activity of the produced compounds was assessed against MCF-7, HepG2, A549, HCT116 cancer cell lines and the results revealed that E III and E IV (a mixture of E I & E III) act as promising anticancer agents in HepG2 and A549 by inhibiting urokinase and histone deacetylase activities.

KEYWORDS: *Anticancer activity; Candida bombicola; Solid state fermentation; Sophorolipids; Soybean oil; Sunflower oilcake*

RESUMEN: ***Producción, caracterización y actividad anticancerígena de soforolípidos producidos mediante fermentación en estado sólido con Candida bombicola de tortas de girasol y aceite de soja.*** Se estudió la producción de soforolípidos por *Candida bombicola* NRRL Y- 17069 cultiva con diferentes técnicas de fermentación en una mezcla de torta de girasol y aceite de soja crudo, como sustratos económicos. El rendimiento más alto (49,5 g·100 g^{-1} de sustrato) se obtuvo por fermentación en estado sólido después de extraer con metanol (IE) seguido de acetato de etilo (EII), y de purificación parcial con hexano (EIII). También se estudió el tiempo de fermentación, considerando que el extracto IE de 12 días mostró una tensión superficial mínima (45 mN·m^{-1}) a una dilución micelar crítica (CMD) de concentración 10 %. Los soforolípidos producidos se caracterizaron y se confirmaron mediante espectroscopia FTIR y RMN de ^{1}H. La actividad anticancerígena de los compuestos producidos se evaluó en células MCF-7, HepG2, A549, líneas celulares de cáncer de HCT116 y los resultados revelaron que EIII y EIV (una mezcla de EI y EIII) actúan como prometedores agentes anticancerígenos en HepG2 y A549 inhibiendo las actividades de uroquinasa e histona desacetilasa.

PALABRAS CLAVE: *Aceite de soja; Actividad anticancerígena; Candida bombicola; Fermentación en estado sólido; Soforolípidos; Torta de girasol*

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1. INTRODUCTION

Biosurfactants are surface-active compounds produced by microorganisms. Most microbial surfactants are complex molecules, comprising of different structures which include lipopeptides, glycolipids, polysaccharide-protein complex, fatty acids and phospholipids (Nitschke and Pastore, 2006). Sophorolipids (SLs), a kind of extracellular biosurfactants, which are produced by yeasts, have been known for a long time, and each of them is comprised of one sophorose molecule, a hydrophilic part, linked to one hydroxyl fatty acid, a lipophilic part by one or two crosslines (Cavalero and Cooper, 2003).

A select number of yeast species, such as *Candida apicola* (Gorin *et al*., 1961), *Candida bogoriensis* (Tulloch *et al*., 1968), *Wickerhamiella domercqiae* (Chen *et al*. 2006a), *Pichia anomala* (Thaniyavarn *et al*., 2008), *Candida guilliermondii* (Sitohy *et al*., 2010), and *Candida bombicola* (Spencer *et al*. 1970, Daverey and Pakshirajan, 2009, Parekh *et al*., 2012) etc., were reported to produce extracellular biosurfactant as SLs.

It is well known that the cost of raw materials generally contributes up to 75% of the selling price of bio-products. Hence, in order to compete with a cheaper chemical surfactant, it is important to use a suitable low-cost fermentation media for the production of SLs. In other words, biosurfactants can replace synthetic ones, which may result in maintaining the cost of the raw materials in its production process at a minimum (Daverey and Pakshirajan, 2009).

Several renewable substrates from various sources, particularly from well-known industrial wastes have been intensively studied for microorganism cultivation and biosurfactant production on a laboratory scale (Daniel *et al*., 1998; Solaiman *et al*., 2004; Felse *et al*., 2007; Thavasi *et al*., 2008; Sitohy *et al*., 2010; Parekh *et al*., 2012).

Worldwide production of oils and fats is about 2.5–3 million tons, 75% of which is derived from plants (Haba *et al*., 2000), which generates great quantities of waste called oil cakes. Annual growth in oil cake production was projected to average 2.3% over the decade to 2010 (Ramachandran *et al*., 2007). Their applications in the field of fermentation technology have resulted in the production of bulk-chemicals and value-added products. (Pandey *et al*., 1999; Vandenberghe *et al*., 2000; Nampoothiri *et al*., 2002; Soccol *et al*., 2005; Roopesh *et al*., 2006; Rashad *et al*., 2012).

In recent years, SLs have attracted much attention for their applications due to their good surface activity, biodegradability, biocompatibility, low toxicity, production process under mild conditions, and production from renewable materials (Lee *et al*., 2008). Also, SLs have attracted more attention since they were found to have good anti-microbial, anti-inflammatory, and even anti-HIV activities, which will broaden their applications in the pharmaceutical sector (Shah *et al*., 2005; Yoo *et al*., 2005; Napolitano 2006). Natural SL molecules and their specific derivatives have recently been reported to have anticancer effects against liver, leukemia, pancreatic, and esophageal cancer cells (Chen *et al*., 2006b; Fu *et al*., 2008, Shao *et al*., 2012). However, little is known about the pharmacological roles of SLs as anticancer drugs. Although there have been great advances in the detection and treatment of cancer, it remains one of the greatest medical challenges. Therefore, the need for better treatments has stimulated research to develop new efficient chemotherapeutic agents for the management of cancer. Urokinase plasminogen activator (uPA) is a serine protease that functions in the conversion of the circulating plasminogen to the active serine protease plasmin. uPA is involved in many physiological functions and it has been implicated in cancer invasion and metastatization (Bdeir *et al*., 2003; Duffy and Duggan, 2004). In addition, Histone deacetylases (HDACs) are a class of enzymes which play an important role in gene expression (Hu *et al*., 2000). Because it has been reported that its inhibition brought about cell-cycle arrest and induced differentiation (Komatsu and Hayashi, 1998), HDAC is considered a target for new types of pharmaceuticals. A possible application of HDAC inhibitors would be the treatment of cancer (Komatsu *et al*., 2001).

Therefore, the objective of the present work was to investigate the production and properties of SLs from *Candida bombicola* cultivated in a mixture of sunflower oil cake and crude soybean oil as alternative unconventional substrates and also, to explore a possible application of the produced SLs in the pharmaceutical field as anticancer agents by studying their *in vitro* cytotoxicity and anti-proliferative activity on four human cell lines.

2. MATERIALS AND METHODS

2.1. Yeast strain

Candida bombicola NRRL Y-17069 was obtained from the Agricultural Research Service, Peoria, Illinois, USA. The culture was kept in a stock slant medium (Wickerman, 1951).

2.2. Substrates

Sunflower seeds (Giza 1) were obtained from the local market (Cairo, Egypt). The seeds were pressed with laboratory-type Carver hydraulic press under 10.000 Ib in^{-2} pressure for 1 h at room temperature according to Üstun *et al*. (1990), then the sunflower

oil cake residue was collected, frozen and kept at −4 °C until analysis. Crude soybean oil was obtained from the Food Technology Research Institute, Soy Processing Center, Agriculture Research Center, Giza, Egypt. All chemicals and reagents used were of analytical grade.

2.3. Inoculum preparation

The inoculum was prepared by transferring a loopful of a stock culture (7 days old) of *C. bombicola* to a 50 ml sterile inoculum medium (Wickerman, 1951), which was then incubated at 30 °C, 180 rpm for 24h.

2.4. Cultivation conditions

2.4.1. Submerged fermentation (medium I)

An aliquot of 1 mL of the inoculum with cell density (1×10^8 cells mL^{-1}) was transferred to an Erlenmeyer flask (250 ml) containing 50 mL of the medium (modified from kim *et al*., 1999) consisting of ($g{\cdot}L^{-1}$) sunflower oil cake waste, 50; soybean oil, 50; NH_4NO_3 1.0; K_2HPO_4, 2.55; NaH_2PO_4, 0.15; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.1; $MnSO_4.H_2O$, 0.02; peptone, 1.0. The final pH was 7.8 and the mixture was incubated for 96 h, at 180 rpm in a controlled incubator shaker (New Brunswick Scientific, USA) at 30 °C.

2.4.2. Submerged fermentation (medium II)

This was done as described above for medium I but without the addition of soybean oil.

2.4.3. Solid state fermentation (medium III)

The solid state fermentation (SSF) medium was prepared according to the method of Ohno *et al.* (1995) with some modifications as follows: 1 mL of the overnight culture was mixed for seeding thoroughly with 5 g of sunflower oil cake waste, 5 g of soybean oil and 4 mL of previously mentioned nutrients (mediumI). Then, the cultures were grown for 96 h in a static incubator at 30 °C.

2.5. Extraction of Sophorolipids

2.5.1. Extraction from submerged medium (media I and II)

The crude SL was isolated from the submerged medium according to kim *et al*. (1999). The fermented product was centrifuged at 15000 rpm for 15 min. and the supernatant was then twice extracted using 2 volumes of ethyl acetate. The extracts were collected and treated with anhydrous sodium sulfate, then the ethyl acetate was evaporated by rotary evaporator and the residue was collected.

2.5.2. Extraction from solid medium

a. Methanol Extraction

As for SSF, the crude SL was isolated according to Ohno *et al*. (1995) by adding 45ml of methanol to one volume of the solid media and the mixture was shaken at 92 strokes min^{-1} for 60 min with a reciprocal shaker (New Brunswick Scientific, USA). The crude extract was then filtered through a 0.20 μm membrane filter (GELMAN sciences, USA), to obtain the methanol extract (E I) (Roongswang *et al*., 2002).

b. Re-extraction by ethyl acetate

A modification was made by re-extracting the fermented substrates (after isolation of EI) with 45mL of ethyl acetate and shaking at 92 strokes min^{-1} for 60 min with a reciprocal shaker, filtered through a 0.20 μm membrane filter to obtain the second ethyl acetate extract (E II). The crude SL was further purified according to Hu and Ju (2001) as follows: the previously obtained ethyl acetate extract (E II) was vacuum-dried at 40 °C to remove the solvent. The residue was twice washed with 10ml hexane to remove any remaining soybean oil, and probably some hydrophobic substances such as fatty acids and alcohols formed in the fermentation. Partially purified SL extract (E III) was thus obtained after vaporizing the residual hexane at 40 °C under vacuum.

2.6. Characterization of Sophorolipids

Surface tension and critical micelle dilution (CMD) were determined with Du-Nouy tensiometer (Kruss type 8451). CMD is defined as the reciprocal of the SL dilution at which a sharp increase in surface tension is observed. To determine the CMD, different serial dilutions were done for the SL extracts (EI, EII, and EIII) obtained from *C. bombicola* (McInerney *et al*., 2004).

2.7. Fourier transform infrared spectroscopy (FTIR)

The infrared (IR) spectrum (from 400 to 4000 wave numbers, cm^{-1}) of SL extracts were recorded using a KBr pellet in Nicolet Impact 6100 FTIR spectrophotometer JASCO, USA.

2.8. NMR spectra analysis

The NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer. ^1H spectra were run at 300 MHz in deuterated chloroform ($CDCl_3$). Chemical shifts are quoted in δ and were related to those of the solvents.

2.9. Cell lines and culturing

Anticancer activity screening for the tested compounds with 4 different human tumor cell lines including breast adenocarcinoma MCF-7; hepatocellular carcinoma HepG2, lung cancer A549 and colon cancer HCT116 which were obtained from the American Type Culture Collection (Rockville, MD, USA). The tumor cells were kept in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (GIBCO), penicillin (100 $U \cdot mL^{-1}$) and streptomycin (100 $\mu g \cdot mL^{-1}$) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells at a concentration of 0.50×10^6 were grown in a 25 cm^2 flask in 5 mL of a complete culture medium.

2.10. *In vitro* Anti-proliferative Assay

The anti-proliferative activity was measured *in vitro* using the 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay according to the previously reported standard procedure (Mosmann 1983). The absorbance was measured with a microplate reader (AsysHitech, Austria) at 570 nm. The relationship between surviving fraction and compound concentration was plotted to get the survival curve for each cell line after the specified time. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated.

2.11. Determination of the level of uPA protein expression

The level of urokinase (uPA) protein expression was determined using AssayMax human uPA ELISA kit (Assaypro, USA) according to manufacturer's instructions. The prepared compounds as well as a standard drug, doxorubicin were incubated for 48 h with, HepG2 and A549 cells at a concentration of 1/10 of the IC_{50} values of each compound.

2.12. Determination of Histone deacetylase (HDAC) activity

Histone deacetylase inhibitors represent a promising new class of compounds for the treatment of cancer (Walkinshaw and Yang, 2008). The activity of HDAC in the lysate of hepatic HepG2 and lung A549 cancer cells treated with the prepared compounds was measured using a colorimetric assay kit (BioVision, Mountain View, kit no. K331-100).

2.13. Statistical analysis

The results are reported as Mean±Standard error (S.E.) for experiments repeated at least four times. Statistical differences were analyzed according to the one way ANOVA test followed by the student's t test wherein the differences were considered to be significant at $p<0.05$.

3. RESULTS AND DISCUSSIONS

3.1. Sophorolipid production

Utilization of a local waste for a maximum production of crude biosurfactant with low cost production materials and processing was the main purpose of this work. Different fermentation techniques were used in this study. By using a submerged medium (medium I), the crude SL obtained reached the level of 20.6 $g \cdot 100\ g^{-1}$ substrate by extraction with ethyl acetate from the substrates (sunflower oil cake plus soybean oil) on the fourth day of fermentation (Figure 1). While a non-significant amount of SL was produced from the same medium without soybean oil (medium II). The yield obtained from the medium I (41.2 $g \cdot L^{-1}$) was higher than that of Davery and Pakshirajan (2010), who found that the maximum yield of *C. bombicola* SL ranged from 23.3 to 33.3 $g \cdot L^{-1}$ using mixed hydrophilic substrates. Also, it was higher than that obtained by Parekh *et al.* (2012) (5.8 $g \cdot 100\ g^{-1}$ substrate) using *C. Starmerella* grown in submerged media containing various lipids and fatty acids obtained from mango kernel. On the other hand, the present yield was slightly lower than that produced by Li *et al.* 2013 (47 $g \cdot L^{-1}$) and Sudha *et al.*, 2010 (49.8 $g \cdot L^{-1}$) using *Wickerhamiella domercqiae* var. and *C. tropicalis* respectively. By using SSF for the same substrates, which consists of sunflower oil cake plus soybean oil (medium III), different extraction methods were examined in one step extraction. Using methanol as extraction solvent, the extract (E I) gave a yield of SL 17 $g \cdot 100\ g^{-1}$ substrate, while the extraction by ethyl acetate gave a yield of 22 $g \cdot 100\ g^{-1}$ substrate. However, using a mixture of methanol and ethyl acetate at a ratio of 1:1 (in one step extraction), the yield was 15 $g \cdot 100\ g^{-1}$ substrate.

On the other hand, employing a new concept for the production process by re-extraction of the remaining fermented media (after isolation of EI) with ethyl acetate (E II) resulted in a higher additional amount of the produced SL (32.5 $g \cdot 100\ g^{-1}$) as shown in Figure 1. This new method of re-extraction produced a higher yield of SL than the traditional extraction method for the solid medium using methanol alone, whereas the two extracts (E I and E II) produced a level of 49.5 $g \cdot 100\ g^{-1}$ for the same substrates on the fourth day of fermentation.

Parekh *et al.* (2012) found that the SL obtained from *C. Starmerella* with SSF of mango kernel was 17.48 $g \cdot 100\ g^{-1}$ substrate after extraction by ethyl acetate, which was lower than our results for ethyl acetate extraction alone (22 $g \cdot 100\ g^{-1}$ substrate).

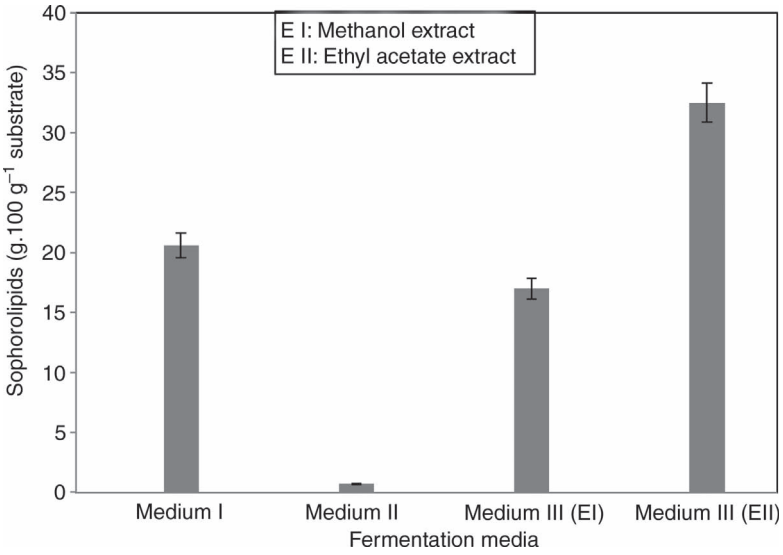

FIGURE 1. The yield of sophorolipids from different fermentation techniques and different solvents on the 4th day of fermentation (data were expressed as mean±S.E. of 4 experiments).

On the other hand, the total yields obtained in this study from methanol extract EI (17 g·100 g^{-1} substrate) and ethyl acetate re-extract EII (32.5 g·100 g^{-1} substrate) were significantly higher (49.5 g·100 g^{-1} substrate).

The highest yield obtained from medium III using our modified re-extraction method was confirmed to be the most suitable extraction method used. From all the above results, it can be concluded that the SSF technique is a suitable technique for further studies with the two steps of extraction.

The course of time of SL production during the SSF (medium III) using the modified extraction method investigated in this study is presented in Figure 2. It can be observed that the methanol extract (E I) productivity was directly proportional with incubation time, whereas the higher yield of SL was obtained on the 16th and 12th days, giving 28.75 and 28.11 g·100 g^{-1} substrate, respectively; while, lower yields were achieved on the 4th and 8th days of incubation (17 and 20.52 g·100 g^{-1} substrate, respectively). However, the ethyl acetate re-extract (E II)

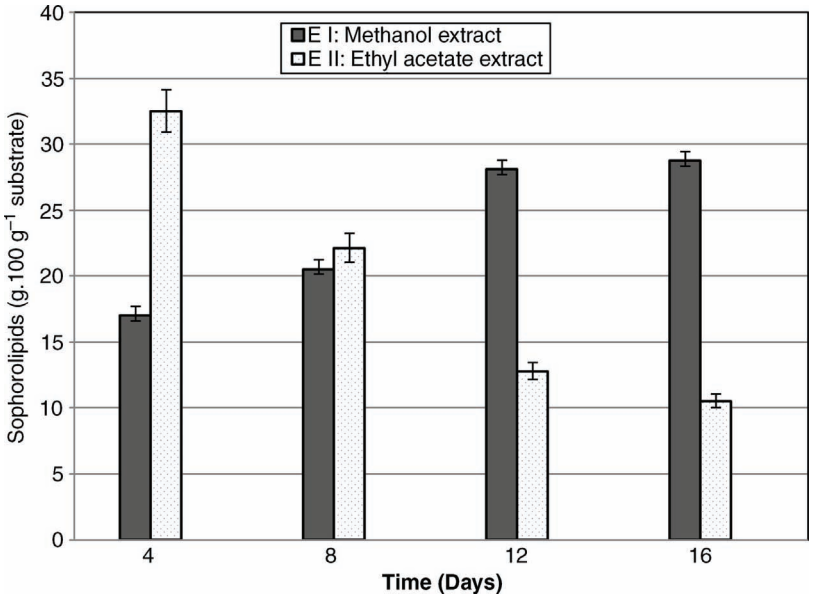

FIGURE 2. The course of time of sophorolipid production during solid state fermentation (medium III) using the modified extraction method investigated (data were expressed as mean±S.E. of 4 experiments).

productivity was inversely proportional with incubation time, where the higher SL yield was obtained on the 4th and 8th days (32.5 and 22.13 g·100 g^{-1} substrate, respectively); subsequently the lower yield was produced on the16th and 12th days of fermentation (10.5 and 12.8 g·100 g^{-1} substrate, respectively). The highest yield obtained from both extracts over the course of fermentation time was 49.5 g·100 g^{-1} substrate on the 4th day, while the lowest was 39.25 g·100 g^{-1} substrate on the 16th day of incubation.

3.2. Identification and characterization of sophorolipids

One of the important characterization properties of a potent surfactant is its ability to lower the surface tension in aqueous solutions. CMD is an indirect indication of surfactant concentration (Makkar and Cameotra, 1997). So, the surface tension (S.T.) and critical micelle dilution (CMD) were determined to evaluate the surface activity of the produced SL resulting from both solvents (E I and E II) during the fermentation period. It was found that the SL extracted with methanol (E I) on the 12th day showed the highest efficiency in reducing the surface tension of water from 70 to 45 mN·m^{-1} when the sample was diluted to 0.1X (10% concentration) which corresponds to its CMD, followed by the ethyl acetate extract (E II) on the same day which gave 48 mN·m^{-1} at the same dilution (0.1X) of 10% concentration CMD. This ethyl acetate extract (E II) was partially purified by hexane to produce the E III extract, which was found to have a non-significant effect on reducing the surface tension (47 mN·m^{-1}) compared to the E II extract at the same CMD (Figure 3). However, the surface tension of the other SL extracts using both solvents during the fermentation period (4–16 days) ranged from 51–56 mN·m^{-1}.

In light of the above results, more studies will be done on the compounds (E I, E II and E III) on the 12th day of fermentation. These results of S.T. for E I & E II nearly coincide with those reported by Kim *et al*. (2005), in that the S.T. of *C. bombicola* SL was 48 mN·m^{-1} when grown in a medium containing glucose and dark soybean oil as carbon sources. However, they were lower than those stated by Amaral *et al.* (2006), who found that the biosurfactant from *Yarrowia lipolytica* has the ability to reduce S.T. by up to 50 mN·m^{-1}

On the other hand, the prepared SL exhibited a higher minimum S.T. than that produced from many *candida* species and ranged from 32–39 mN·m^{-1} (Sobrinho *et al*., 2008; Daverey and Pakshirajan 2009, 2010; Chandran and Das 2011).

3.3. FT-IR analysis

The FT-IR spectra of the prepared SL compounds (E I, E II and E III) showed mostly identical absorption bands (Figure 4). The FT-IR spectra for all extracts revealed broad bands at 3385–3424 cm^{-1} which correspond to the O-H stretch, while asymmetrical stretching (v_{as} CH_2) and symmetrical stretching (v_s CH_2) of methylene occurs at 2926 cm^{-1} and 2855 cm^{-1} respectively. Absorption bands at (1742–1745 cm^{-1}) contribute to C=O stretching from lactone ester or acids. The C=O absorption band from acetyl esters was observed at 1238–1240 cm^{-1} in all structures, while the bands at 1460 cm^{-1} corresponded to the C-O-H in the plane binding of carboxylic acid (-COOH) in the structure of the three products. The stretch of C-O band of C (-O)-O-C in lactones exists in all products at 1164–1170 cm^{-1}, also the C-O stretch from C-O-H groups of sugar (sophorose moiety) is observed at 1080–1098 cm^{-1}; finally the IR spectra revealed the absorption band

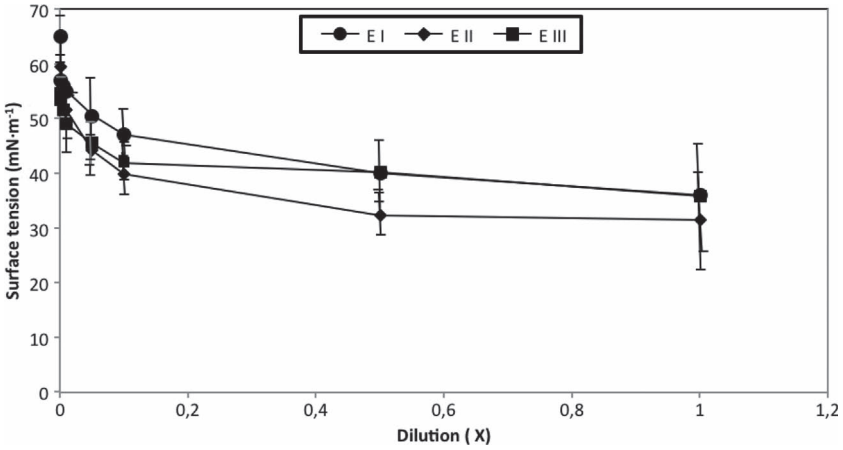

FIGURE 3. The surface tension and critical micelle dilution (CMD) of the produced sophorolipid extracted by methanol (E I), ethyl acetate (E II) and purified by hexane to form ethyl acetate extract (E III). The critical micelle dilutions (CMD) are marked in circles (data were expressed as mean±S.E. of 4 experiments).

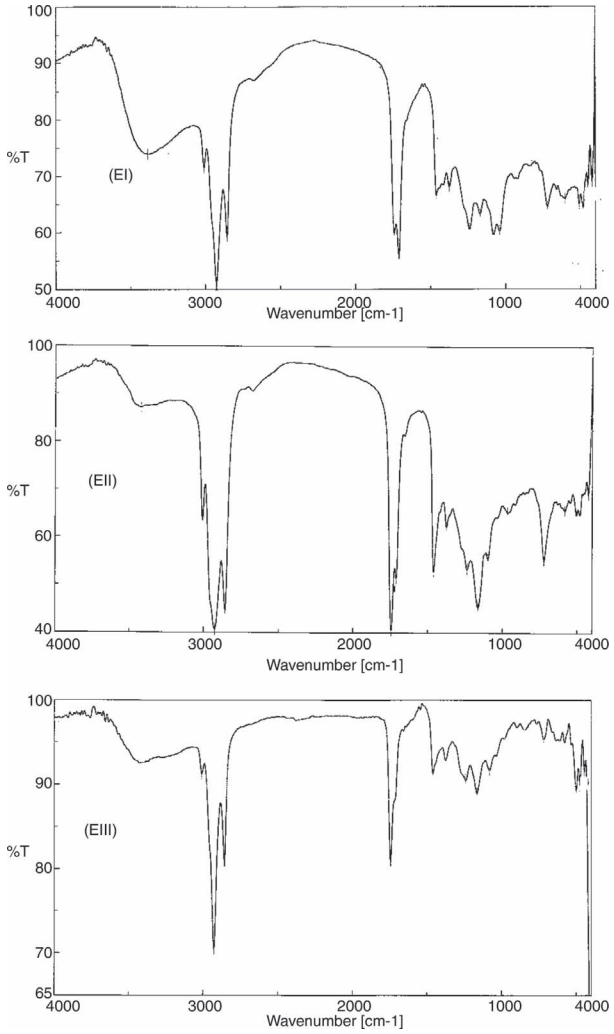

FIGURE 4. FTIR spectra of the produced sophorolipid compounds E I, E II and E III.

for C=C at 719–723 cm^{-1} in all the studied structures. All these structural details of the products were found similar to those reported by Yongmei and Lu-Kwang (2001); Daverey and Pakshiragan (2009) and Bajaj *et al.* (2012), which confirmed that these products are SL compounds.

3.4. ^{1}H NMR analysis

The structure of the produced SL compounds (E I, E II and E III) was elucidated using ^{1}H NMR analysis. The spectra of the three extracts were assigned to a typical glycolipid-type structure. In all compounds, the signals of the vinyl group (–CH=CH–) were at 5.32≈5.39 ppm, while the signals at 2.08 ppm were denoting the presence of–CH_3 group. Multiple peaks from 1.23 to 1.31 ppm put the accent on the existence of fatty acid chain moiety in all structures. The presence of the–CO-CH_3 group in all structures was confirmed by the signals at 2.06 ppm in the spectra; on the other hand, the protons of two glucose were resonated at 3.4≈4.3 ppm in the Hexane fraction (E III). The signals obtained were similar to the one in the ^{1}H NMR spectrum of the SL compounds reported by other researchers (Wei *et al.*, 2005; Chen *et al.*, 2006a; Gao *et al.*, 2007; Daverey and Pakshiragan 2009 and Bajaj *et al.*, 2012).

According to the results of the FT-IR and ^{1}H NMR analyses, all these structural features further confirmed the SL group of compounds (acidic and lactone ring form) in the produced biosurfactants.

3.5. *In vitro* anti-proliferative activity

The anti-proliferative activity of the produced crude and partially purified SLs (E l, E II and E III) and a mixture of E I and E III (E IV) was evaluated against breast adenocarcinoma MCF-7; human hepatocellular carcinoma HepG2, human lung cancer A549 and human colon cancer HCT116 cell lines using an MTT colorimetric assay, in comparison with doxorubicin as reference drug. The results revealed that all compounds did not exert any activity against human breast cancer MCF-7 or colon cancer HCT116 cell lines.

The anti-proliferative activities are expressed by median growth inhibitory concentration (IC_{50}) and provided in Table 1. From the results it is evident that the tested SLs compounds displayed potent growth inhibitory activity against only two tested cell lines (HepG2 and A549).

The IC_{50} of compounds E l, E II, E III and E IV for the HepG2 cell line was 47.00±4.20, 36.00±3.50, 23.70±2.63 and 19.00±1.80 $\mu g \cdot mL^{-1}$ respectively. It is clear that, while the IC_{50} of E III (23.70±2.63 $\mu g \cdot mL^{-1}$) is close to the value of the reference drug, doxorubicin (20.10±2.00 $\mu g \cdot mL^{-1}$), the mixture of the 2 extracts (E IV) was more potent (19.00±1.80 $\mu g \cdot mL^{-1}$) than the doxorubicin. Similarly, the IC_{50} of SL compounds E I, E II, E III and E IV for the A549 cell line was 46.60±4.30, 41.20±4.60, 29.00±2.80

TABLE 1. Cytotoxicity (IC_{50}, $\mu g \cdot mL^{-1}$) activity of the produced sophorolipid compounds (E I, E II, E III and E IV) against four human malignant cell lines as measured with the MTT assay method

	Cell line			
Compound	**HepG2**	**MCF-7**	**A549**	**HCT116**
Doxorubicin	20.10±2.00	24.00±2.50	25.50±2.70	19.25±2.00
E I	47.00±4.20	NA	46.60±4.30	NA
E II	36.00±3.50	NA	41.20±4.60	NA
E III	23.70±2.63	NA	29.00±2.80	NA
E IV	19.00±1.80	NA	25.77±2.20	NA

Data are expressed as means±S.E. of four separate experiments.
NA: no activity.

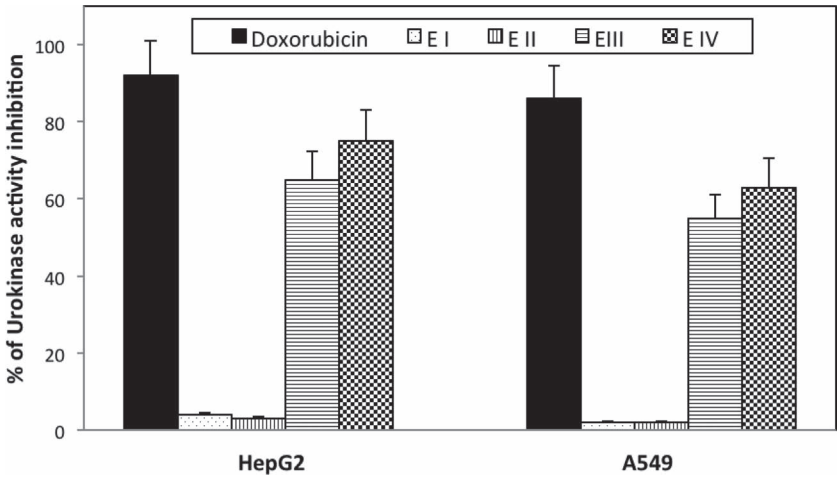

FIGURE 5. The percent of uPA inhibition of the produced sophorolipid compounds (E I, E II, E III and E IV) comparing to doxorubicin in HepG2 and A549 cell lines (data were expressed as mean±S.E. of 4 experiments).

and 25.77±2.20 $\mu g \cdot mL^{-1}$ respectively. It is clear that the mixture (E IV) was more potent (25.77±2.20 $\mu g \cdot mL^{-1}$) than doxorubicin (25.50±2.70 $\mu g \cdot mL^{-1}$).

3.6. The level of uPA protein expression

To identify the mechanism of action responsible for the cytotoxicity of the prepared SL compounds, the level of urokinase (uPA) protein expressed in the two cell lines (HepG2 and A549) were estimated quantitatively.

In the case of the hepatic cancer cell line HepG2 and lung cancer cell line A549, the SL compounds E I and E II have no effect on the expression of uPA, only 4 and 3% inhibition were obtained in HepG2, while 2% and 2% inhibition were obtained in A549 cells. On the other hand, the level of uPA expression decreased in SL compounds E III and E IV by the following percent, E III (65, 55%) and E IV (75, 63%) in HepG2 and A549, respectively. From the results, the sophorolipid compound E IV exhibited a good anticancer activity in HepG2 and A549 similar to doxorubicin (92% and 86%, respectively) (Figure 5). In both HepG2 and A549 the inhibition of uPA activity of the tested SLs compounds was in accordance with the cytotoxicity activity.

3.7. Histone deacetylase (HDAC) activity

Histone deacetylase (HDAC) inhibitors are a new class of targeted anticancer agents which are potent inducers of growth arrest, differentiation, and/or apoptotic cell death of transformed cells *in vitro* and *in vivo* (Bi and Jiang, 2006). Thereby, we examined

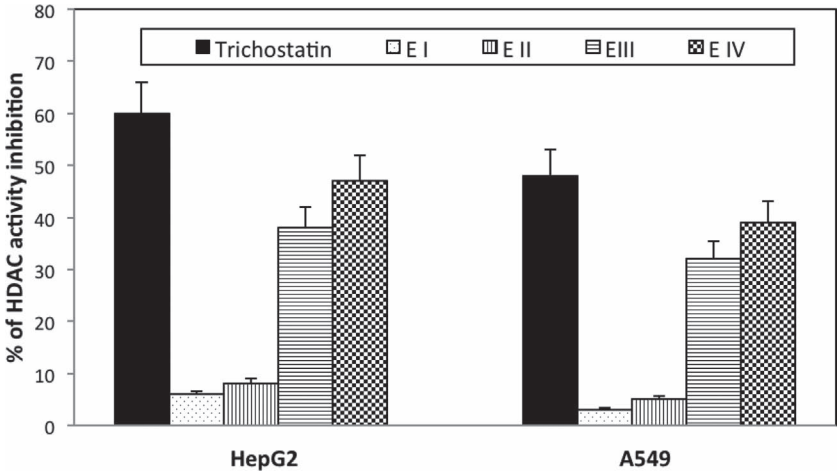

FIGURE 6. The effect of the prepared sophorolipid compounds (E I, E II, E III and E IV) on HDAC activity in HepG2 and A549 cell lines after treatment with the prepared compounds and cells with Trichostatin, as a known HDAC inhibitor. The data was compared with the HDAC activity of the control cancer cells (data were expressed as mean±S.E. of 4 experiments).

the effect of the prepared SL compounds as histone deacetylase inhibitors in an attempt to use them in tumor treatment.

In this work the activity of HDAC in the lysate of hepatic HepG2 and lung A549 cancer cells treated with the prepared SL compounds as well as Trichostatin, as a known inhibitor, was measured and the data were calculated as percentage of inhibition as compared to the control, untreated cancer cells. While the treatment of hepatic HepG2 and lung A549 cancer cells with Trichostatin resulted in 60% and 48% inhibition, respectively, as compared with the control untreated cancer cells, the treatment with compounds E l, E II, E III and E IV resulted in 6, 8, 38 and 47%, respectively in hepatic HepG2 cells. Similarly, the treatment of lung A549 cells with all the prepared SL compounds (E I-E IV) resulted in the inhibition of the activity of HDAC by 3, 5, 32 and 39%, respectively (Figure 6).

Taken together, these findings suggest that there are correlations between the cytotoxicity of the tested SL compounds and inhibition of the urokinase and Histone deacetylase activities. The tested compounds exert anti-carcinogenic activity in hepatic HepG2 and lung A549 cancer cell lines by lowering the activity of these enzymes which may reduce the cell proliferation and result in significant growth inhibition. The potent anticancer effect of the compound E IV needs further investigation.

Chen *et al.* (2006b) studied the cytotoxic effects of the SL produced by the yeast *Wickerhamiella domercqiae* on cancer cells of liver, lung and leukemia (H7402, A549, HL60 andK562). The results showed a dose-dependent inhibition ratio on cell viability according to the drug concentration ≤62.5 $\mu g \cdot mL^{-1}$, which suggested that the SL produced by *W. domercqiae* have anticancer activity.

Fu *et al.* (2008) have demonstrated that acidic SL had stronger cytotoxicity (35.5% cytotoxicity at 100 mg mL concentration) than lactonic SL (26.2% cytotoxicity at 100 $mg \cdot mL^{-1}$ concentration) against pancreatic cancer cells and he reported that these different results are probably attributed to the inhibition mechanism of SL on pancreatic cancer cells, which are different from other cancer cells. Also, Shao *et al*. (2012) investigated the bioactivity of SLs with different structures against human esophageal cancer cells, and the results revealed that the SLs with different unsaturation degrees of hydroxyl fatty acids had different cytotoxic effects on esophageal cancer cells.

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