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INHIBITORY ACTIVITIES OF MICROALGAL EXTRACTS AGAINST EPSTEIN-BARR VIRUS (EBV) ANTIGEN EXPRESSION IN LYMPHOBLASTOID CELLS

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Abstract – The inhibitory activities of microalgal extracts against the expression of three EBV antigens, latent membrane protein (LMP)1, Epstein-Barr nuclear antigen (EBNA)1 and Z Epstein-Barr reactivation activator (ZEBRA) were assessed by immunocytochemistry. The observation that the methanol extracts and their fractions from *Ankistrodesmus convolutus*, *Synechococcus elongatus* and *Spirulina platensis* exhibited inhibitory activity against EBV proteins in three Burkitt's lymphoma cell lines at concentrations as low as 20 μg/ml suggests that microalgae could be a potential source of antiviral compounds against EBV.

Keywords: microalgae; Epstein-Barr virus (EBV); *Ankistrodesmus convolutes; Synechococcus elongatus*

INTRODUCTION

Algae are a potential source of yet to be fully explored antiviral compounds. The sulfated polysaccharides from the red algae *Porphyridium* sp.*,* and the blue green algae *Spirulina platensis* were found to inhibit the replication of herpes simplex virus-1 and -2 (HSV-1 and HSV-2), varicella zoster virus (VZV) and human immunodeficiency virus type 1 (HIV-1) (Hayashi et al., 1996; Huleihel et al. 2001; Huleihel et al., 2002; Barron et al., 2008). The compounds griffithsin and fucoidan isolated from the red seaweed *Griffithsia* sp. and brown algae, respectively, were found to inhibit the entry of human immunodeficiency virus-1 (HIV-1) into host cells (Wang and Ng 2001; Hayashi et al., 2008; Hidari et al., 2008; Micewicz et al., 2010). Despite many antiviral studies on algal compounds, there are few reports about the effects of these compounds against the Epstein-Barr virus (EBV).

The EBV is an etiological factor in Burkitt's lymphoma (BL) and other EBV-related malignancies, such as nasopharyngeal carcinoma, Hodgkin's disease and infectious mononucleosis (Rickinson and Kieff, 2006; Rezk and Weiss, 2007). During latent infection, several of the nine viral latent gene products, such as the Epstein-Barr nuclear antigen

1 (EBNA1) and latent membrane proteins 1 (LMP1), are expressed to transform and immortalize lymphoblastoid cell lines (Farrell 1995; Thorley-Lawson et al., 2008). LMP1 plays a crucial role in enhancing cell proliferation and providing an oncogenic effect by activating nuclear factor (NF) kappa B (κB) which upregulates anti-apoptotic genes (Fries et al., 1996; Luftig et al., 2004). On the other hand, EBNA1 activates the initiation of EBV DNA replication in host cells, and partitions the viral episomes during cell division for long-term plasmid maintenance (Hung et al., 2001). EBNA1 also confers resistance to apoptosis by upregulating the apoptotic suppressor protein gene (Lu et al., 2011). The reactivation of EBV from latency is initiated via activation of the expression of the immediate-early BZLF1 gene that encodes for the Z Epstein-Barr replication activator (ZEBRA) or Zta (Countryman and Miller, 1985).

At present, there is no specific approved drug against EBV (Gershburg and Pagano, 2005). In clinical applications, non-EBV specific antiviral drugs are used to treat EBV-associated diseases. Acyclovir is used to inhibit replication of the virus by targeting DNA polymerase (Elion, 1993). Hence, there is great interest in exploring natural compounds for their anti-EBV potential. To date, several molecules that display anti-EBV properties have been isolated from higher plants. Epigallocatechin gallate from green tea, auraptene and umbelliprenin from *Ferula* sp., resveratrol from grapes, as well as the ethanol extracts from *Polygonum cuspidatum* and *Chrysanthemum indicum*, inhibit the EBV lytic cascade by inhibiting EBV proteins (Chang et al., 2003; Iranshahi et al., 2008; Yiu et al., 2010; Yiu et al., 2011; Kim et al., 2012).

Our previous report showed the microalgae also contained bioactive anti-EBV compounds (Kok et al., 2011). The methanol extracts from *Ankistrodesmus convolutus*, *Synechococcus elongatus* and *Spirulina platensis* were found to inhibit the release of EBV from BL cell lines, Akata, B95-8 and P3HR-1. We investigated whether these algal extracts inhibited the release of cell-free EBV after viral antigen expression in BL cells. The objective of this study was to assess the inhibitory activities of methanol extracts and their fractions from *Ankistrodesmus convolutus*, *Synechococcus elongatus* and *Spirulina platensis* against the expression EBV proteins EBNA1, LMP1 and ZEBRA in lymphoblastoid cells.

MATERIALS AND METHODS

Microalgae cultures

Three strains of microalgae were obtained from the University Malaya Algae Culture Collection (UMACC): *Spirulina (Arthrospira) platensis* UMACC 161, *Synechococcus elongatus* UMACC 105 and *Ankistrodesmus convolutus* UMACC 101 (Phang and Chu, 1999). *Spirulina platensis* and *Synechococcus elongatus* were grown in Kosaric medium. and *Ankistrodesmus convolutus* UMACC 101 was grown in Bold's Basal Medium (BBM) (Nicols and Bold, 1965; Zarrouk, 1996). The cultures were placed on shelves illuminated with fluorescent lamps (42 μmol/m^2/s, 12:12 h light-dark cycle) in a controlled-temperature (28°C) room.

Extract preparation

The algal extracts were prepared as described previously (Kok et al., 2011). A bioassay-guided fractionation approach was used to test the antiviral activity of the extracts. Only extracts and fractions that exhibited strong inhibitory activities against the release of cell-free EBV shown in the previous study were further tested for their inhibitory activities against the expression of EBV antigens in BL cells (Table 1). The concentrations tested were much lower than the values for the inhibition concentration $(IC)_{50}$ obtained previously. Cells treated with commercial antiviral drugs such as acyclovir and foscarnet (Sigma) (0-100 μg/ml) served as positive controls.

Lymphoblastoid cell cultures

Three EBV-positive Burkitt's lymphoma (BL) cell lines were used: Akata, B95-8, and P3HR-1. All cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with

Table 1. Microalgal extracts and fractions used for testing against the expression of EBV antigens in the three Burkitt's Lymphoma cell lines. The extracts and fractions were selected based on their positive activities against the release of cell-free EBV DNA.

Extract/Fraction	Yield (μg/mg dry weight)	Cell Lines		
		Akata	B95-8	P3HR-1
Crude extracts				
Ankistrodesmus convolutus (AC) methanol extract	178.3	+	+	+
Synechococcus elongatus (SC) methanol extract	177.5	+	+	+
Spirulina platensis (SP) methanol extract	86.9	+	+	+
Column chromatography fractions				
ACF2	20.4	+	-	-
ACF3	23.2	+	-	-
ACF1	7.2	-	+	-
ACF5	8.3	-	+	-
ACF6	22.8	-	+	-
SEF1	12.0	-	+	+
SEF3	3.9	-	+	+
HPLC subfractions				
ACF2'a	171	+	-	-
ACF2'c	144	+	-	-
ACF2'd	89	+	-	-
ACF2'e	50	+	-	-
ACF2'f	20	+	-	-
ACF1'a	95	-	+	-
ACF1'b	38	-	+	-
SEF1'a	149	-	-	+
SEF1'c	315	-	-	+
SEF1'd	66	-	-	+
SEF3'a	54	-	-	+
SEF3'c	182	-	-	+

+ expression of viral proteins tested; - expression of viral proteins were not tested; F fractions collected from silica gel column; ' subfractions collected from HPLC column

10% (v/v) fetal bovine serum (FBS) and 50 IU penicillin-streptomycin, and grown in a CO_2 incubator. B95-8 and P3HR-1 cells were chemically induced using 0.2% (v/v) phorbol 12-myristate-13-acetate (32 μM) and 1% (v/v) sodium N-butyrate (0.3 μM). Akata cells were induced into a lytic cycle with 0.8% (v/v) rabbit antiserum to human IgG (ICN Pharmaceuticals, USA).

Immunocytochemistry analysis

After 72 h of incubation with the microalgal extracts, the BL cells were washed twice with phosphate buffer saline (PBS). A small drop of the suspension (10^6 cells/ml) was smeared on a Teflon-coated multi-welled slide and dried fixed with cold acetone. The fixed slides were boiled in 10 mM citrate buffer, pH 6.0 for 4 min and rinsed with distilled water. Hydrogen peroxide (3%) was added to the wells and then washed with distilled water followed by PBS.

Monoclonal mouse anti-Epstein-Barr virus antibodies, LMP1 clone S12 (BD Bioscience, USA), EBNA1 (Calbiochem, USA) and BZLF1 protein, ZEBRA clone BZ.1 (DakoCytomation, Denmark) were diluted to 1:100 with PBS containing 10% normal goat serum before addition to the samples in the wells. The samples were left at 37°C for 4 h to allow hybridization to occur. The slides were then washed in PBS thrice (5 min each). HRP-conjugated anti-mouse polymer (DakoCytomation, Denmark) was added to the wells and left at 37°C for 1 h. The cells

were washed in PBS with slow shaking for 5 min. The substrate 3,3-diaminobenzidine (DAB) (DakoCytomation, Denmark) was used for color development. Unstained cells were counter-stained with hematoxylin. After washing, the excess hematoxylin, the slides were dipped in ammonia water (10%) for 10 s. The dried slides were mounted using Depax and covered with cover slips. The slides were examined under the light microscope (Nikon Eclipse 80i). A minimum of 400 cells were scored. The percentage of cells expressing the targeted of protein was calculated using the following equation:

Percentage of cells that express the targeted protein (%) = number of positively stained cells x 100%
Total number of cells counted in a population

Statistical analysis

Data are presented as the means with standard deviation derived from duplicate samples from two independent experiments. The data were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range test using Statistica software (Version 5). A p value < 0.05 was regarded as statistically significant.

RESULTS

Inhibitory activity of the methanol extracts

The inhibitory effects of the microalgal extracts against the expression of EBV viral proteins were assessed by determining the number of cells expressing the proteins in a cell population, estimated by immunocytochemistry. Cells that expressed the antigens showed positive binding and stained brown compared to the background that appeared blue. The percentage of positively stained cells in the treated groups was compared to the negative control.

Different BL cell lines expressed different levels of EBV antigens. The percentages of Akata cells expressing EBNA1, LMP1 and ZEBRA in the negative control population were 35.6%, 18.1% and 32.5%, respectively (Fig. 1). In Akata cells, of the three EBV proteins studied, the number of ZEBRA positive Akata cells was decreased the most, from 32.5% to less than 7%. The methanol extract from *Ankistrodesmus convolutus* (100 μg/mL) reduced the percentage of cells expressing ZEBRA most effectively, from 32.5% to 3.3% (Fig. 1c).

The percentages of EBNA1 and LMP1 positive B95-8 cells were relatively low compared to Akata and P3HR-1 cells (Figs. 2a, 2b). The methanol extract from *Synechococcus elongatus* (100 μg/ml) caused a reduction of EBNA1 positive BL cells by more than 60% in all three BL cells. The microalgal extracts reduced the number of ZEBRA positive B95-8 cells from 25% to less than 8.5% (Fig. 2c).

In P3HR-1 cells, the percentage of LMP1 positive cells compared to non-treated cells was reduced by 66% after the treatment with 100 μg/ml of the methanol extract from *Spirulina platensis* (Fig. 3b). In contrast, 100 μg/ml of the methanol extract from *Ankistrodesmus convolutus* and *Synechococcus elongatus* decreased the percentage of ZEBRA positive cells by more than 66% (Fig. 3c). In addition, the methanol extract from *Spirulina platensis* (100 μg/mL) reduced the percentage of EBNA1 positive P3HR-1 cells by 75% when compared to the negative control.

All three microalgal methanol extracts decreased the percentages of LMP1 positive BL cells by 46-69% when compared to the non-treated group. The microalgal methanol extracts reduced the expression of ZEBRA more than 50% in all three Akata, B95-8 and P3HR-1 cell lines.

Inhibitory activity of silica gel fractions

Some of the methanol extracts exhibited inhibitory activity after fractionation. The fraction ACF2 (20 μg/mL) reduced EBNA1 positive Akata cells by 70%, whereas the crude methanol extract of *Ankistrodesmus convolutus* did not display any inhibitory activity against EBNA1 (Fig. 4a).

As shown in Fig. 5, ACF1, ACF5, ACF6, SEF1 and SEF3 did not significantly ($p>0.05$) reduce the

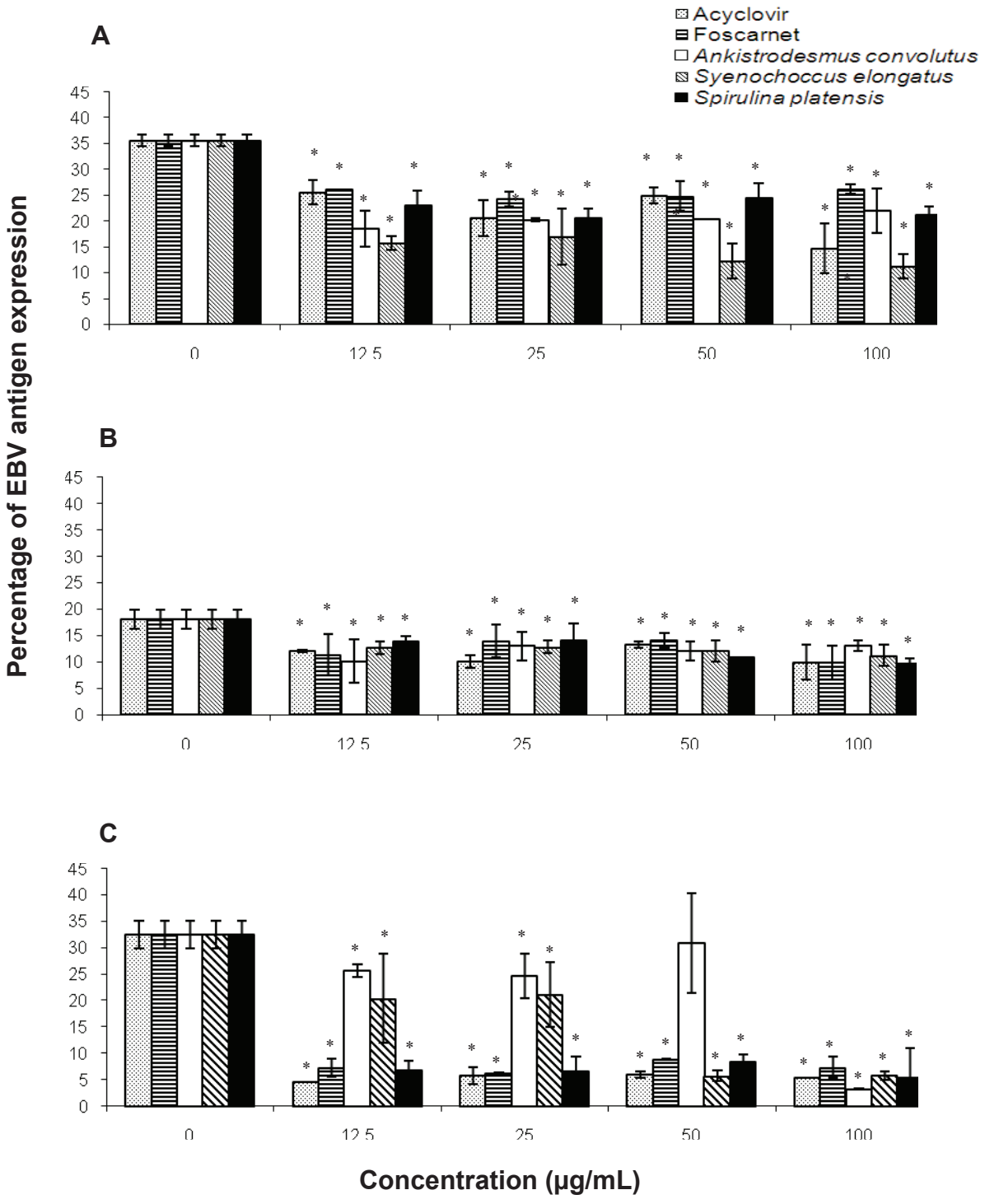

Fig. 1. Percentage of EBV antigen-positive Akata cells after treatments with the extracts for 72 h, determined by immunocytochemistry. The percentage of positively staining cells relative to the whole cell population was determined as described in the Materials and Methods. A) EBNA1; B) LMP1; C) ZEBRA. Values shown are means of two independent experiments performed in duplicate. Asterisks show significant differences relative to the control ($p < 0.05$).

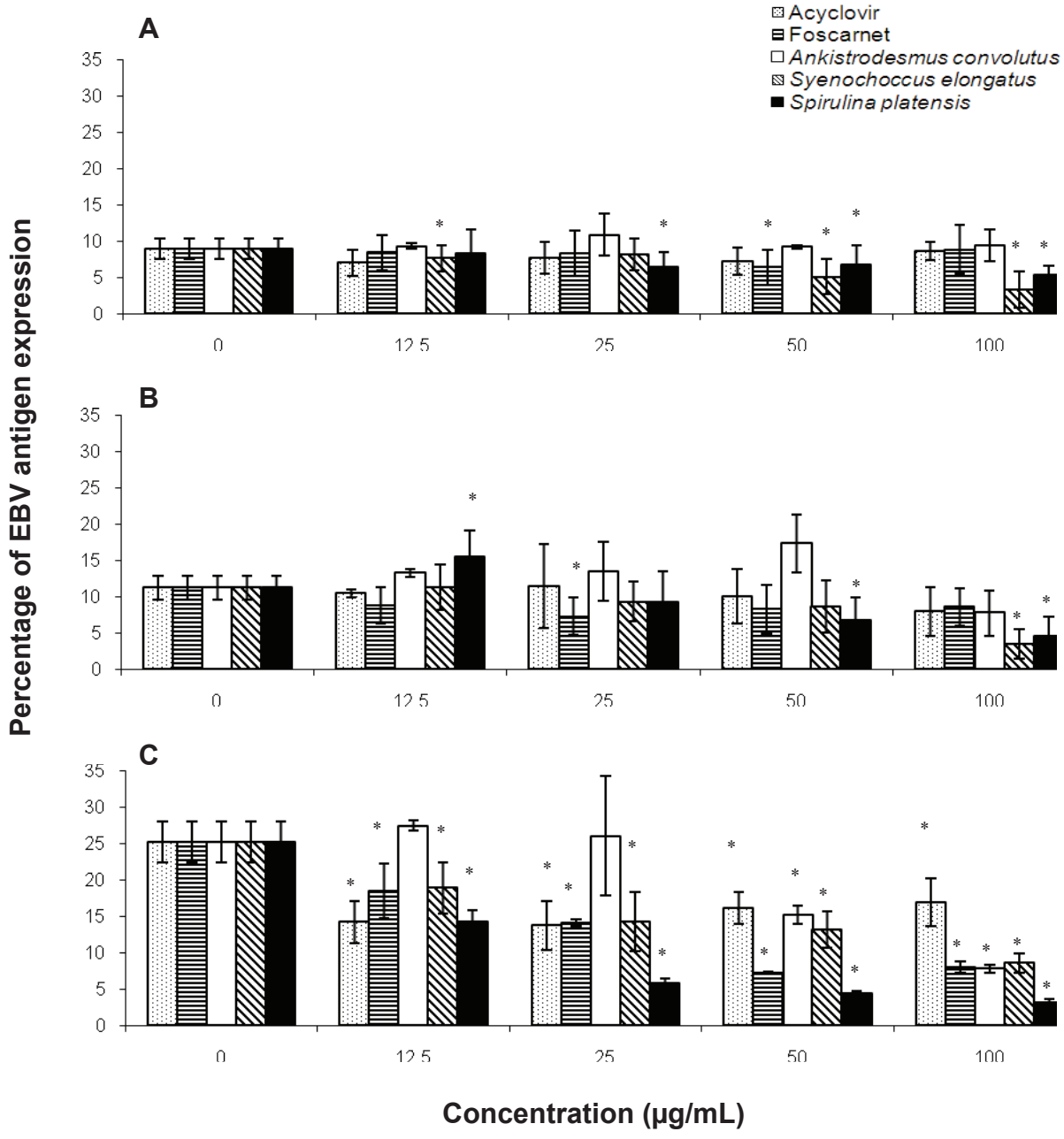

Fig. 2. Percentage of EBV antigen positive B95-8 cells after treatment with extracts for 72 h, determined by immunocytochemistry. The percentage of positive-staining cells relative to the whole cell population was determined as described in the Materials and Methods. A) EBNA1; B) LMP1; C) ZEBRA. Values shown are means of two independent experiments performed in duplicate. Asterisks show significant differences relative to the control ($p < 0.05$).

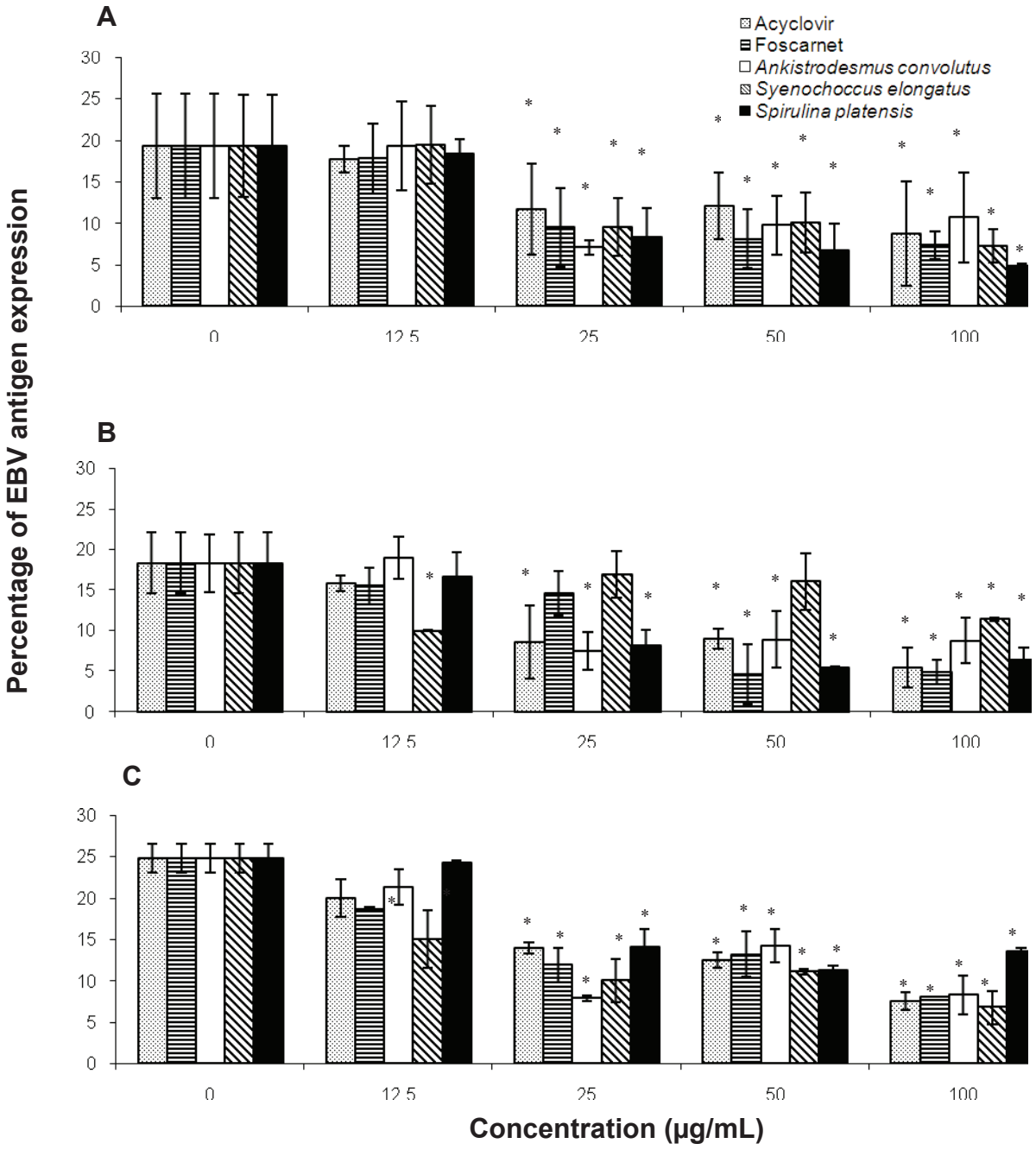

Fig. 3. Percentage of EBV antigens positive P3HR-1 cells treated with microalgae extracts for 72 determined by immunocytochemistry. The percentage of positive-staining cells was determined as described in the Materials and Methods. A) EBNA1; B) LMP1; C) ZEBRA. Values shown are means of two independent experiments performed in duplicate. Asterisks show significant difference relative to the control at ($p < 0.05$).

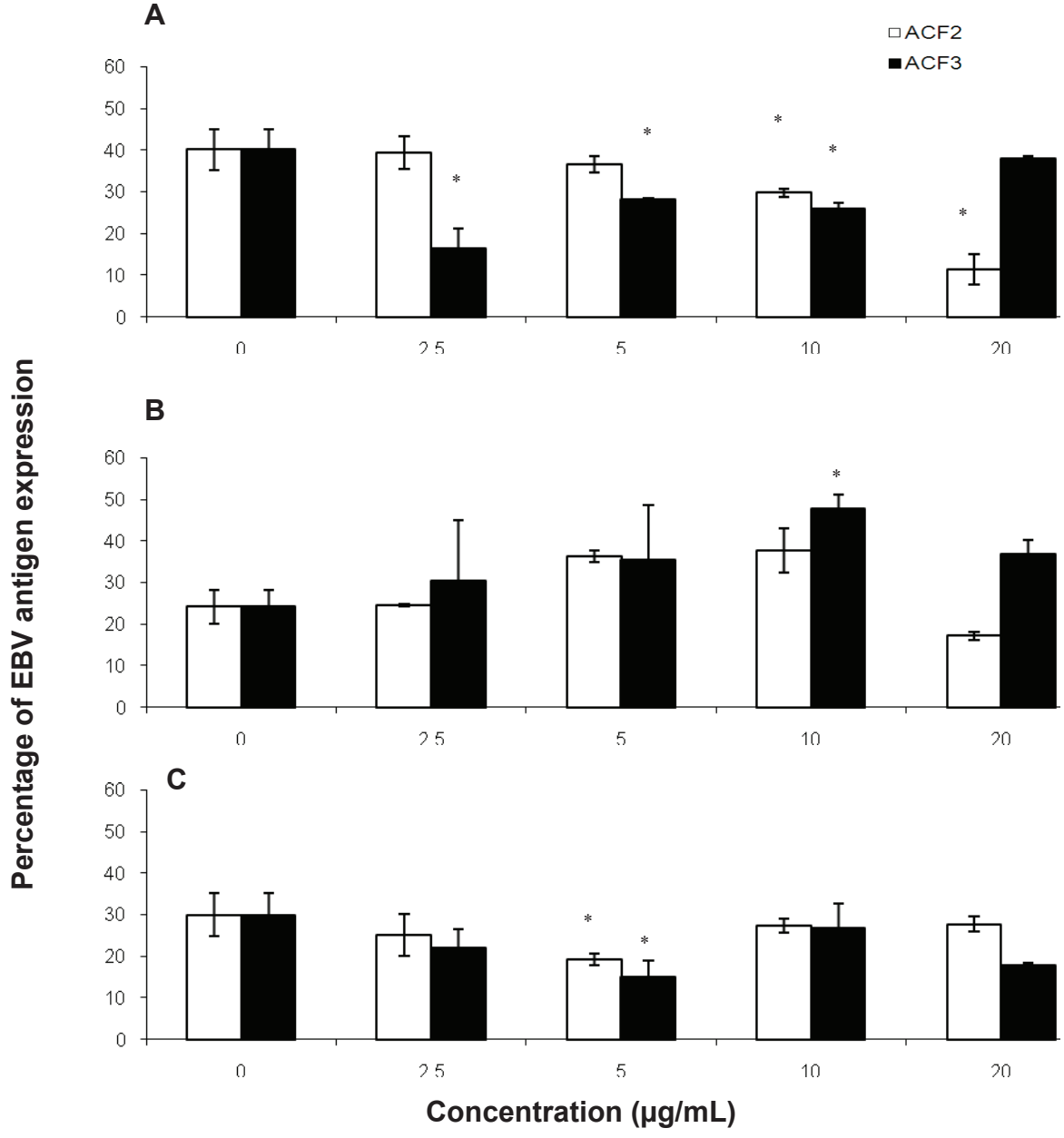

Fig. 4. Percentage of EBV antigen positive Akata cells after treatments with fractions (obtained after column chromatography) of the methanol extracts from *Ankistrodesmus convolutus*(AC), ACF2 and ACF3 for 72 h, determined by immunocytochemistry. The percentage of positive-staining cells was determined as described in the Materials and Methods. The background was stained with hematoxylin. A) EBNA1; B) LMP1; C) ZEBRA. Values shown are means of two independent experiments performed in duplicate. Asterisks show significant differences relative to the control ($p< 0.05$).

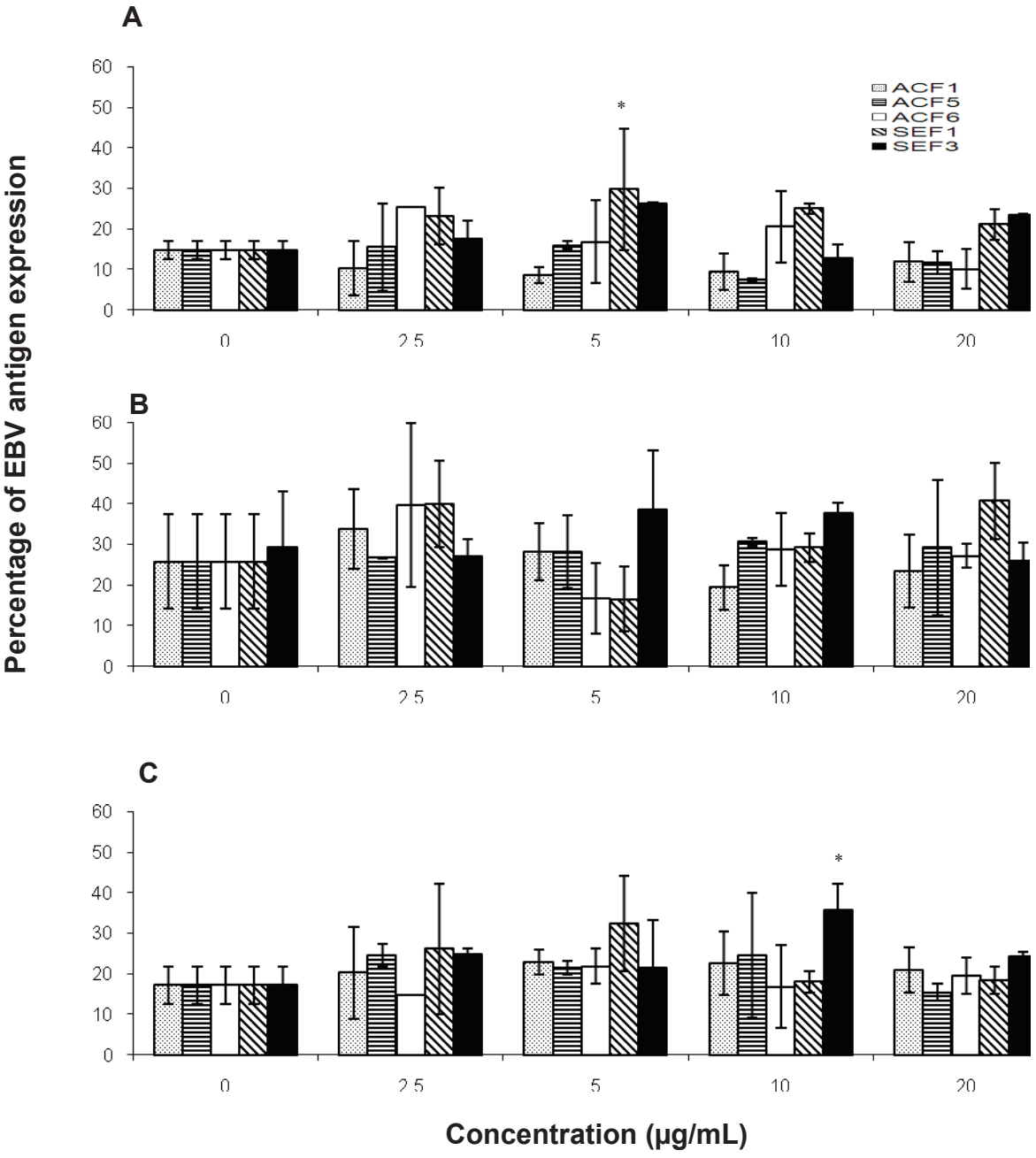

Fig. 5. Percentage of EBV antigens positive B95-8 cells treated with fractions of the methanol extracts (obtained after column chromatography) from *Ankistrodesmus convolutus*(AC) and *Synechococcus elongatus* (SE) ACF1, ACF5, ACF6, SEF1 and SEF3 for 72 h, determined by immunocytochemistry. The percentage of positive-staining cells was determined as described in the Materials and Methods. The background was stained with hematoxylin. A) EBNA1; B) LMP1; C) ZEBRA. Values shown are means of two independent experiments performed in duplicate. Asterisks show significant differences relative to the control ($p < 0.05$).

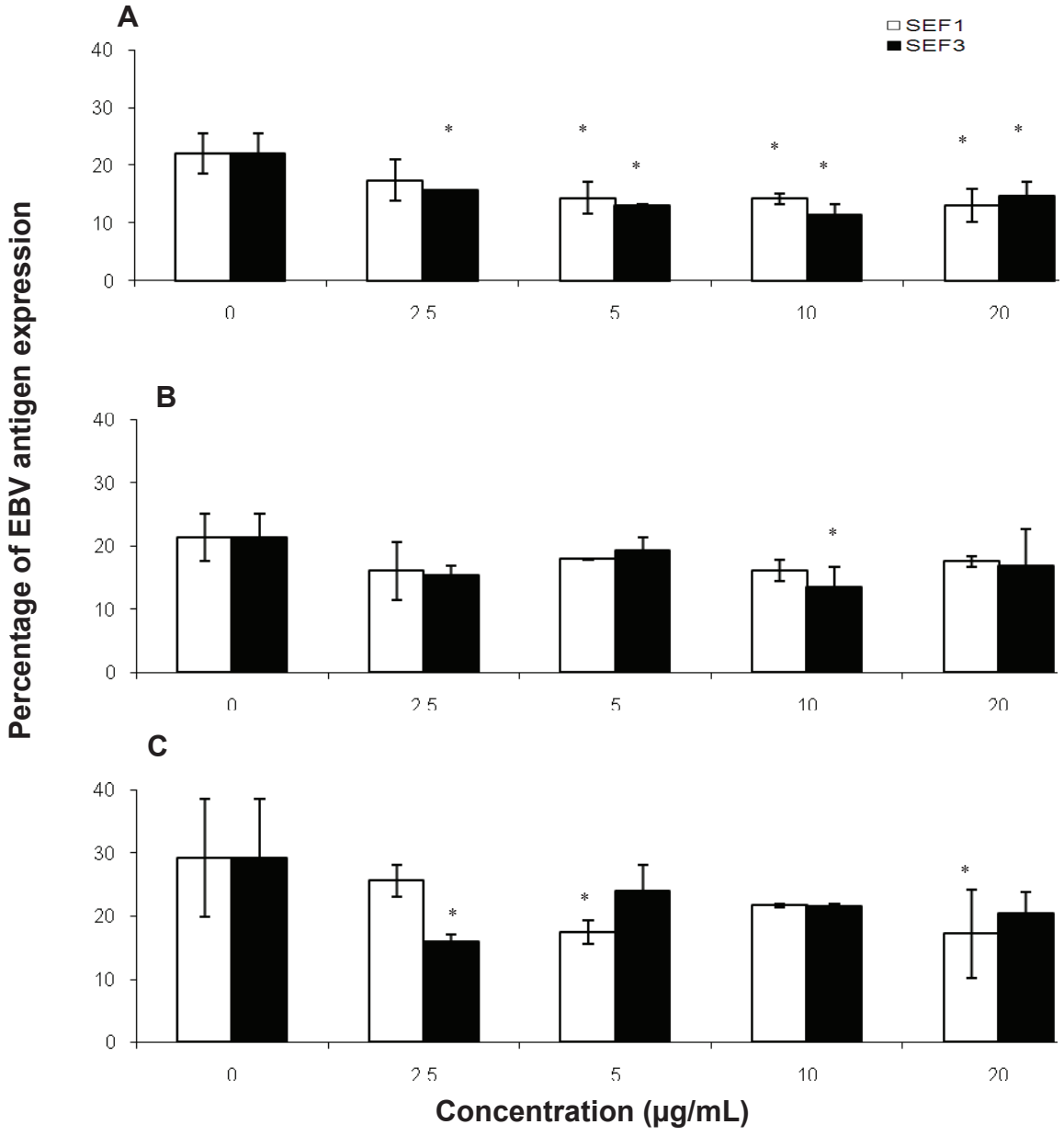

Fig. 6. Percentage of EBV antigens positive P3HR-1 cells treated with fractions of the methanol extracts (obtained after column chromatography) from *Ankistrodesmus convolutus*(AC) and *Synechococcus elongatus* (SE) SEF1 and SEF3 for 72 h, determined by immunocytochemistry. The percentage of positive-staining cells was determined as described in the Materials and Methods. The background was stained with hematoxylin. A) EBNA1; B) LMP1; C) ZEBRA. Values shown are means of two independent experiments performed in duplicate. Asterisks show significant differences relative to the control ($p< 0.05$).

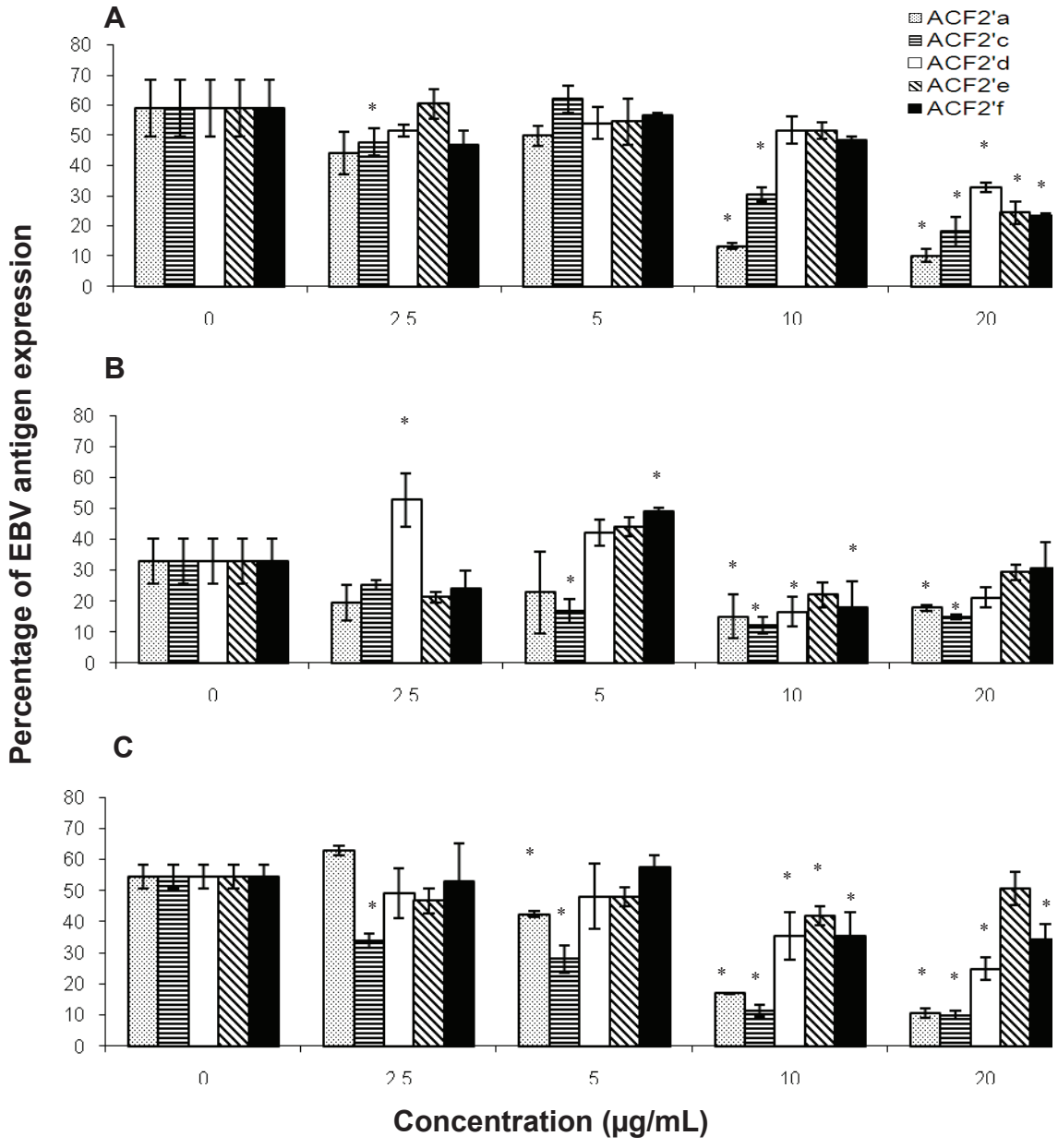

Fig. 7. Percentage of EBV antigens positive Akata cells treated with HPLC subfractions of ACF2- ACF2'a, ACF2'c, ACF2'd, ACF2'e and ACF2'f for 72 h, determined by immunocytochemistry. The percentage of positive-staining cells was determined as described in the Materials and Methods. The background was stained with hematoxylin. A) EBNA1; B) LMP1; C) ZEBRA. Values shown are means of two independent experiments performed in duplicate. Asterisks show significant differences relative to the control ($p < 0.05$).

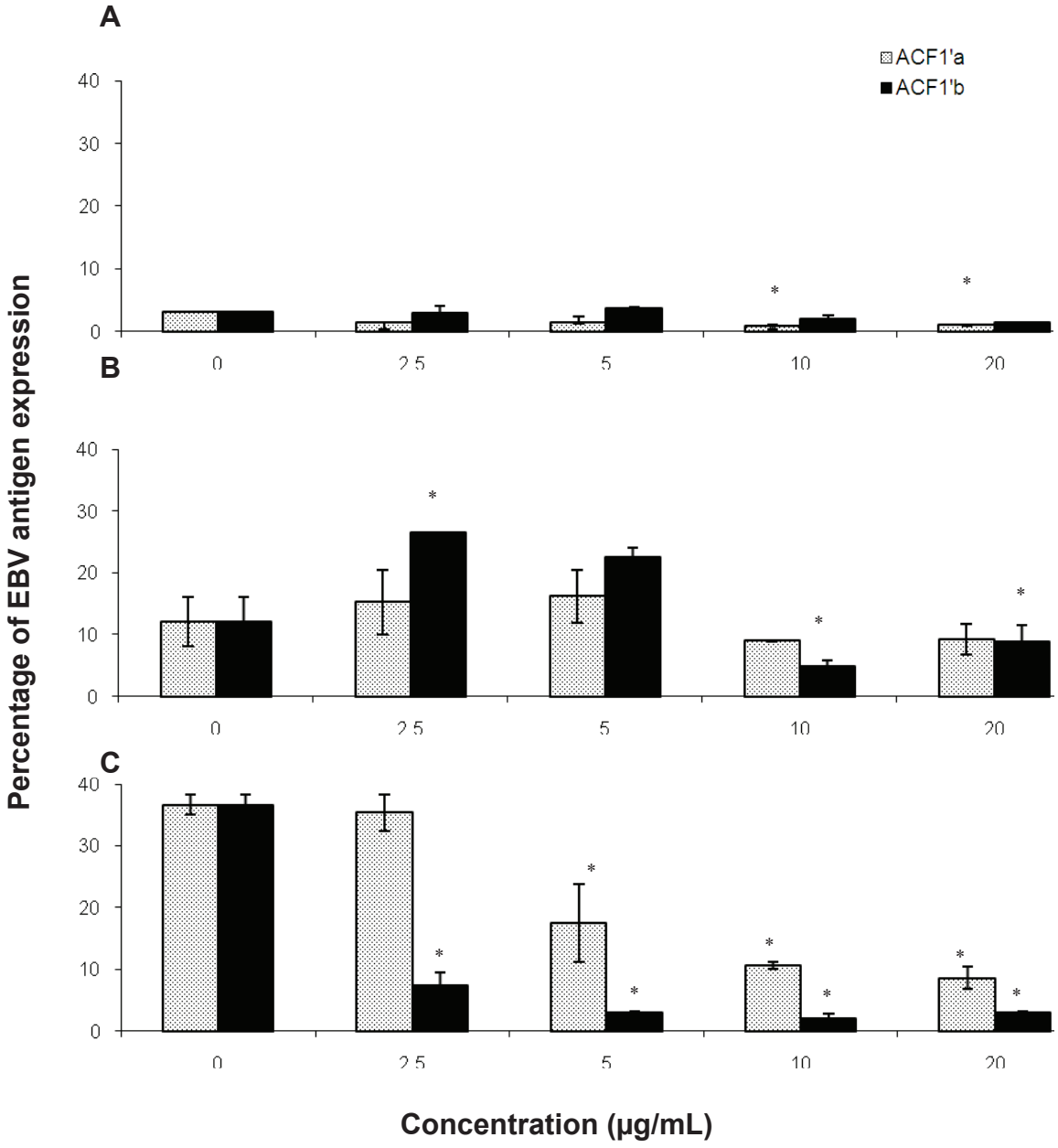

Fig. 8. Percentage of EBV antigens positive B95-8 cells treated with HPLC sub-fractions of ACF1- ACF1'a and ACF1'b for 72 h, determined by immunocytochemistry. The percentage of positive-staining cells was determined as described in the Materials and Methods. The background was stained with hematoxylin. The background was stained with hematoxylin. A) EBNA1; B) LMP1; C) ZEBRA. Values shown are means of two independent experiments performed in duplicates. Asterisks show significant differences relative to the control ($p< 0.05$)..

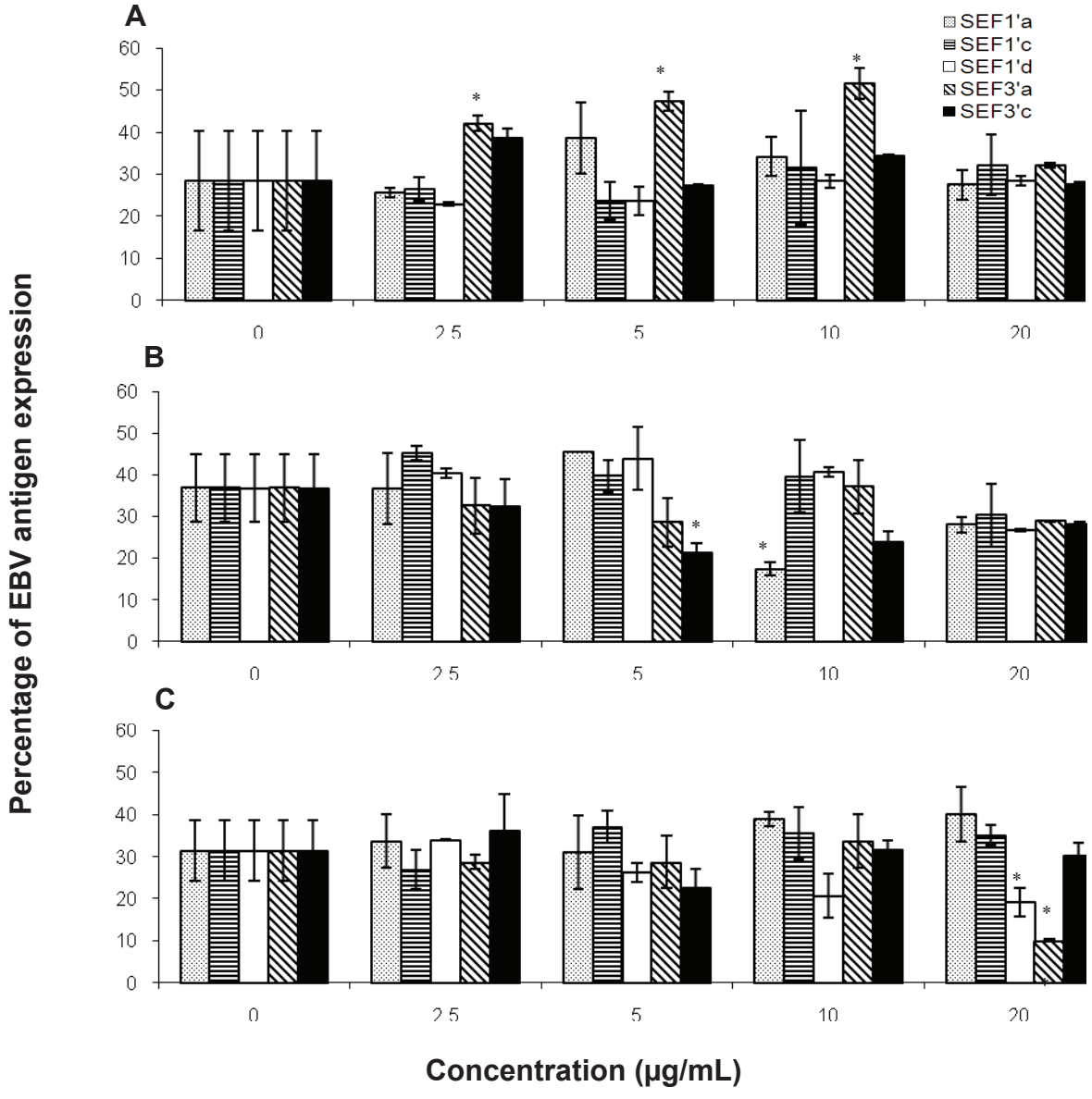

Fig. 9. Percentage of EBV antigens positive P3HR-1 cells treated with HPLC sub-fractions of SEF1- SEF1'a, SEF1'c, SEF1'd, SEF3'a and SEF1'c for 72 h, determined by immunocytochemistry. The percentage of positive-staining cells was determined as described in the Materials and Methods. The background was stained with hematoxylin. A) EBNA1; B) LMP1; C) ZEBRA. Values shown are means of two independent experiments performed in duplicate. Asterisks show significant differences relative to the control ($p < 0.05$).

expression of the three antigens in B95-8 cells, although they showed antiviral activity in the real time PCR assay in our previous work (Kok et al., 2011). SEF3 reduced significantly ($p>0.05$) the number of EBNA1 positive P3HR-1 cells at 2.5-20 μg/ml, while SEF1 (20 μg/ml) reduced the number of EBNA1 and ZEBRA positive P3HR-1 cells by 40.4% and 41.4%, respectively. (Figs. 6a and 6c, respectively).

Inhibitory activities of HPLC subfractions

The HPLC subfractions of ACF2 showed different activities against the expression of the three antigens in Akata cells (Fig. 7). The bioactivities of ACF2'a, ACF2'c, ACF2'd, ACF2'e and ACF2'f were highest at 20 μg/ml, as they reduced by 45-83% the number of EBNA1 positive Akata cells as compared to the control (Fig. 7a). All the HPLC sub-fractions (10 μg/ml) significantly reduced ($p<0.05$) the percentage of LMP1-positive Akata cells by 45-63% (Fig. 7b). ACF2'a and ACF2'c at 10 μg/ml caused a considerable 65% reduction in the number of ZEBRA positive Akata cells (Fig. 7c). Clearly, the inhibitory activities in *Ankistrodesmus convolutus* against ZEBRA were preserved after fractionation.

The HPLC subfractions of the extract from *Ankistrodesmus convolutus*, ACF1'a and ACF1'b, significantly reduced ($p<0.05$) the number of ZEBRA-positive B95-8 cells, but not EBNA1- and LMP1-containing cells (Fig. 8). Nevertheless, the percentage of ZEBRA-positive B95-8 cells was lower after treatment with ACF1'b compared to ACF1'a (Fig. 8c). None of the of the HPLC subfractions of the extracts of *Synechococcus elongatus* were capable of lowering the expression of the three antigens in P3HR-1 cells (Fig. 9). The only active HPLC subfraction that showed remarkable inhibition activity was SEF3'a (20 μg/ml), which reduced the number of ZEBRA-positive P3HR-1 cells by 67.8% compared to the control (Fig. 9c). Overall, the subfractions from the microalgal extracts, ACF1'a, ACF1'b, ACF2'a, ACF2'c, SEF1'd and SEF3'a, seemed to be more active in inhibiting the expression of the EBV lytic protein ZEBRA in BL cells (Figs. 7c, 8c, 9c).

DISCUSSION

The microalgal extracts tested herein previously showed inhibitory activity against the release of cell-free EBV DNA. Our aim was to examine further the antiviral effects of the the extracts. Generally, they showed inhibitory activities against the expression of EBV proteins, observed as a reduction in the numbers of cells expressing the EBV proteins. The persistency, replication and gene expression of EBV is dependent on the binding of EBNA1 to the origin of plasmid replication (OriP), which serves to maintain the EBV episome in dividing cells (Hung et al., 2001). Therefore, EBNA1 is an attractive target for potential EBV inhibitors because of its critical role in EBV-associated disorders. In this study, the methanol extract from *Ankistrodesmus convolutus*, *Synechococcus elongatus* and *Spirulina platensis* (100 μg/mL) exhibited a significant reduction of EBNA1-positive BL cells (by more than 60%). These microalgal extracts could decrease the presence of EBV episomes in new daughter cells and terminate latent EBV infection by inhibiting EBV episome replication. Persistence of the EBV genome and the lytic cascade can be obstructed via downregulation of the latency-associated promoter Qp (Kieff and Rickinson, 2006). This causes fewer EBV virions to be assembled and released. In our previous study where we assessed the copy number of cell-free EBV DNA, ACF2 and its HPLC subfractions reduced the number of cell-free EBV DNA by 50% at concentrations between 1.6-4.2 μg/ml (Kok et al., 2011).

Recently, LMP1 was proposed as a therapeutic target in EBV-associated disorders (Hannigan and Wilson, 2010). The microalgal crude extract exhibited >30% inhibitory activity against LMP1 in all three cell lines, except *Ankistrodesmus convolutus* in B95-8 cells. These compounds could block the NF-κB pathway and disrupt cell survival, leading to cell death (Lavorgna and Harhaj, 2012). This is in agreement with our previous study where the HPLC subfractions ACF2'c and ACF2'd reduced the number of viable Akata cells by about 50% (Kok et al., 2011). We also speculate that the reduction of LMP1 activates the DNA repair pathway in host cells, leading to deg-

radation of EBV viral DNA and the resulting release of lower amounts of cell-free EBV. This was shown in our previous work where the methanol crude extracts, ACF2'a, ACF2'c, ACF2'd, ACF2'e and ACF2'f, inhibited the release of cell-free EBV DNA by 50% at concentrations lower than 20 µg/ml.

All three microalgal extracts and some of their HPLC subfractions lowered the number of ZEBRA-positive cells by about 60% in all three Akata, B95-8 and P3HR-1 cell lines. ZEBRA is a crucial lytic transactivator in the EBV life cycle. Therefore, these extracts could switch off the lytic transactivation cycle in BL cells by blocking the activation of the BZLF1 gene, thus preventing the switch from latent to lytic cycle gene expression. Consequently, this leads to a decrease in the numbers of assembled and released viral particles (Feederle et al., 2000; El-Guindy et al., 2006). This explains the lower copy number of cell-free EBV released by the host cell after exposure to the methanol extract of *Ankistrodesmus convolutus* that was reported previously.

The mechanisms responsible for the induction of lytic EBV are complex and involve calcium-dependent mechanisms, protein kinase C (PKC), p38, c-jun N-terminal kinase (JNK) and mitogen activated protein kinases (MAPK) (Israel and Kenney, 2003). Beside BZLF1, BRLF1 mediates the switch form latent to lytic viral replication (Miller and El-Guindy, 2002). Therefore, the effects of the microalgal extracts on the cell cycle of BL cells warrants further studies.

CONCLUSION

Methanolic microalgal extracts and their subfractions obtained after HPLC reduced the number of LMP1-, EBNA1- and ZEBRA-positive BL cells, suggesting that these preparations could serve as sources of potential anti-EBV agents.

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