Antiproliferative effects of fluorine substitute 3,5-di-*tert*-butylphenol bearing Schiff bases using CFSE-based cell proliferation assay

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The determination of antiproliferative properties of compounds on tumour cells is important for assessment of their efficacy in cancer treatment. CFSE-labelled K562 cells were incubated with doxorubicin and *ortho*- or *para*-fluorosubstitute Schiff bases (compounds 1 and 2 respectively). CFSE intensities were analysed using flow cytometry. K562 cells treated with doxorubicin resulted in homogeneous high intensity fluorescence after 96 h of incubation. Schiff bases exhibited antiproliferative effects, but lower than doxorubicin. Our results reveal that CFSE assay can be used for determining *in vitro* antiproliferative features of anticancer drugs and/or compounds from herbal or chemical sources.

Keywords: Cancer, CFSE, doxorubicin.

THERE have been many attempts to explore new chemotherapeutic agents from chemical and herbal sources worldwide. The determination of cytotoxic and antiproliferative effects of organic compounds has crucial importance in the evaluation of anticancer potential[1]. Although there are some precise methods such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or ATP-based tumour chemosensitivity (ATP-TCA) assays to determine the cytotoxic capacity of herbal or chemical compounds[2–4], there are no precise methods to quantitively determine the antiproliferative effects of compounds or assess multiparameters in anticancer research.

The carboxyfluorescein diacetate succinimidyl ester (CFSE) assay is currently used to determine cell proliferation with flow cytometry[5]. The CFSE assay is based on general protein labelling by CFSE in which an amino-reactive dye forms stable covalent bonds with cell proteins[6–8]. An equal and progressive division of CFSE fluorescence occurs within daughter cells after each cellular division which suggests *in vitro* cell proliferation[9]. This fluorescent cell tracking assay can be a powerful tool to study the antiproliferative effects of anticancer drugs or herbal and chemical compounds *in vitro*.

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Schiff bases derived from an amine and carboxylic compounds belong to a class of ligands involved in the coordination of metal ions via azomethine nitrogen[10]. The C=N linkage is crucial for biological activity in azomethine derivatives, and it was reported that various azomethines are characterized to have roles against bacteria, fungi and cancer[11]. An appreciable amount of Schiff-base complexes are quite successful models of biological compounds[12].

Fluorinated compounds have recently drawn attention due to their therapeutic applications in various medical areas[13]. 5-Fluorouracil (5-FU), a synthetic flourinated antineoplastic agent, is being used as an antimetabolite to treat malignancies such as breast, head, neck and gastrointestinal malignancies[14]. 5-FU irreversibly inhibiting thymidylate synthase can cause the death of rapidly dividing cancerous cells[15]. Tamoxifen is another fluorinated compound used for treatment of hormone-dependent breast cancer as an oestrogen antagonist[16]. Gemcitabine is another fluorinated compound used in the treatment of some cancers[17].

In the present study our aim is to determine the antiproliferative properties of fluorinated Schiff bases on a K562 myelomonocytic leukemic cell line using a CFSE assay, which could provide considerable quantitative data.

All chemicals and solvents were used as received. *N,N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO), acetonitrile (MeCN), ethanol, 2-fluoroaniline, 4-fluoraniline, 2,4-di-*tert*-butylphenol, hexamethylenetetramine, acetic acid, H_2SO_4 were purchased from Sigma-Aldrich and used without further purification. 3,5-di-*tert*-butylsalicylaldehyde was prepared in accordance with published procedure[18].

N-2-fluorophenyl-3.5-di-*tert*-butylsalicylaldimine (compound **1**) and *N*-4-fluorophenyl-3.5-di-*tert*-butyl-salicylaldimine (compound **2**) were prepared using the standard Schiff base condensation reaction with equimolar amounts of 3,5-di-*tert*-butyl-salicylaldehyde and appropriate fluoroaniline in ethanol at reflux for 5–6 h in the presence of 3–4 drops of formic acid and recrystallized from methanol. The Schiff base ligand, 2-hydroxyacetophenonehydrazone of 2-hydroxybenzoylhydrazine (L2SH2) was prepared by reacting salicylhydrazide (1.52 g/10 mmol) and 2-hydroxy acetophenone (1.36 g/10 mmol) in stirred ethanol (25 ml) for 3 h. The resulting white compound was filtered and washed thrice with ethanol and dried over fused $CaCl_2$. All Schiff bases were obtained as yellow products. The melting point and IR, UV/*Vis* and 1H NMR data of these compounds were in agreement with earlier reported data[19].

5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester (Sigma, USA) was dissolved in DMSO and diluted in phosphate-buffered saline (PBS) under sterile conditions. K562 cells were incubated with 10 μg/ml CFSE for 10 min at 37°C under humidified 5% CO_2 atmosphere.

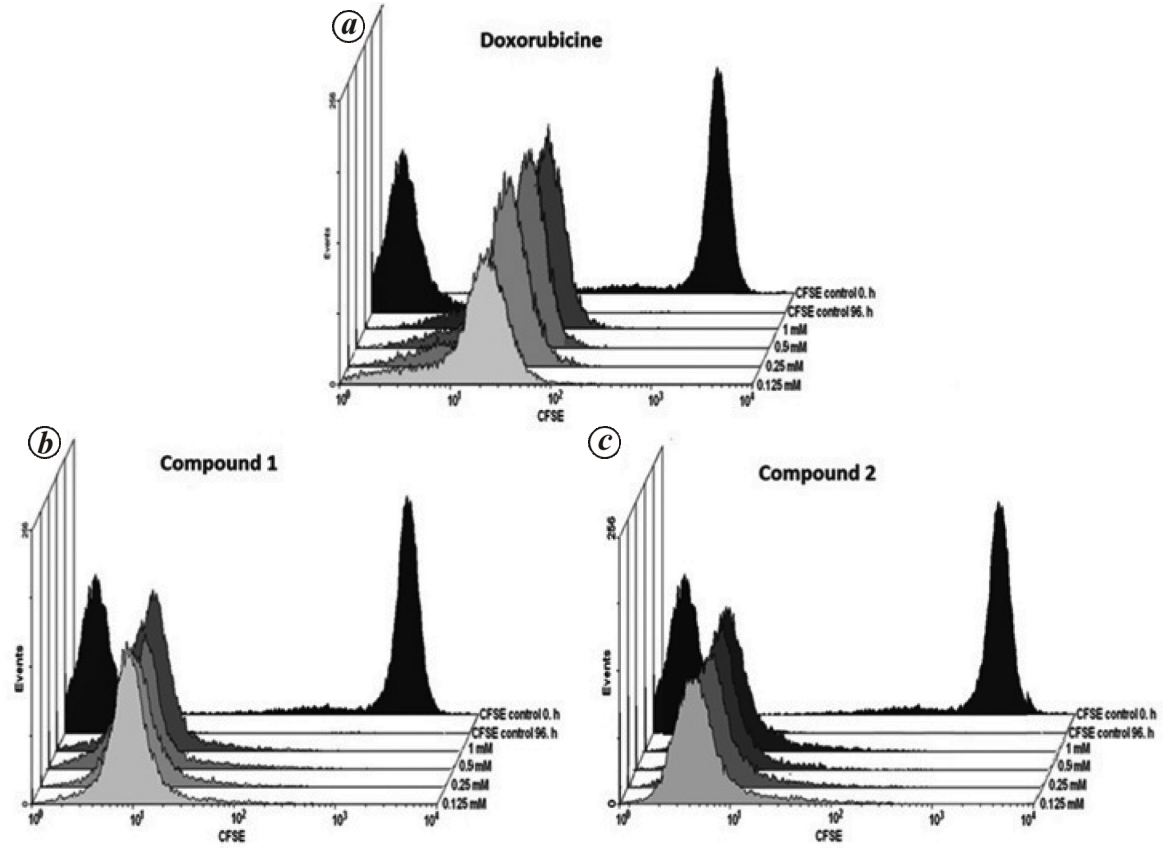

Figure 1. Fluorescent intensity of CFSE-labelled K562 cells after 96 h incubation.

After incubation, the cells were washed thrice and analysed to determine the level of cellular staining using flow cytometry.

The chemicals used were diluted and placed into 96-well culture plate in triplicate order at doses of 1, 0.5, 0.25 and 0.125 mM. The CFSE stained K562 cells were then seeded into culture plate wells at a dose of 10^4 cells/100 ml in RPMI1640 medium, supplemented with 10% FCS and antibiotics. Doxorubicin was used as a positive control with the doses mentioned above. Culture plates were incubated for 96 h at 37°C under humidified 5% CO_2 atmosphere. The maximum proliferation of K562 cells was monitored using three untreated wells.

After 96 h of incubation with doxorubicin and Schiff bases, CFSE-labelled K562 cells were analysed using flow cytometry to determine the cells' CFSE level. Unstained K562 cells were used to determine autofluorescence. CFSE stained cells were analysed on a FacsCalibur flow cytometer (BD, FacsCalibur, USA) supplied with an argon-ion laser tuned at 488 nm. K562 cells were totally gated at FS and SS histogram. The level of CFSE cellular staining was analysed at FL1 histogram (green fluorescence channel) in the range of 10^0–10^4. Forward- and side-scatter and fluorescence signals were determined for each cell, and the data were acquired in list mode.

Data list mode files were analysed using Cellquest and FCS Express 4 Flow Research Edition softwares. The CFSE level of the K562 cells was analysed in the histogram plot to show the dose-dependent antiproliferative activities of the doxorubicin and *ortho*- or *para*-fluorosubstitute Schiff bases (Figure 1). Proliferation analyses were carried out with 1 mM dose of doxorubicin and *ortho*- or *para*-fluorosubstitute Schiff bases. The rates of new generations and proliferation of K562 cells were analysed using a proliferation plot from the FCS Express 4 program (Table 1 and Figure 2).

Doxorubicin, and *ortho*- or *para*-fluorosubstitute Schiff bases were diluted and placed into 24-well culture plate in triplicate order at doses of 1, 0.5, 0.25 and 0.125 mM, and the K562 cells were then seeded into culture plate wells at a dose of 10^5 cells/ml in RPMI1640 medium, supplemented with 10% FCS and antibiotics. Untreated cells were used as control. Culture plates were incubated for 96 h at 37°C under humidified 5% CO_2 atmosphere in a cell culture incubator. Cell viability was evaluated with a Beckman Coulter Vi-Cell XR cell viability analyser, using trypan blue exclusion method.

After 96 h of incubation, doxorubicin treated cells (0.1–1 mM) showed an intensity level of fluorescence higher than untreated cells (Figure 1). The flourescence intensity of the 1 mM doxorubicin treated cells was

Table 1. Antiproliferative effects of doxorubicine and *ortho*- or *para*-fluorosubstitute Schiff bases

	% of cells			Original cells (%)	Mean		Median channel	Peak		Proliferation index	R Chi square*
	Fit	Noise	Undivided		Geometric	Arithmetic		Value	Channel		
Control (0 h)	72.4	28.3	72.4	72.4	1161	1441	1485	1582	1582	1.0	1.14
Control (96 h)	0	97.2	NA	NA	2.2	10.2	2.0	818	1.0	NA	195.8
Doxorubicine (96 h)	89.0	14.1	0	13.3	24.0	39.0	37.1	246	46.0	6.6	1.3
Compound **1** (96 h)	91.9	13.0	0	2.6	8.5	17.8	8.0	159	8.8	35.2	0.9
Compound **2** (96 h)	94.8	11.5	0	2.6	6.3	12.5	5.4	143	4.6	34.3	1.1

*Values under 5 indicate a good fit. The mean number of original cells that existed prior to any division. The arithmetic mean channel number of the events that were accepted by the gating formula that fell within the specified marker. The median channel shows the value of events that were accepted by the gating formula that fell within the specified marker. Peak value shows the number of events that were in the peak channel. The peak channel indicates the greatest numbers of events that were located. PI is the average number of cells that an initial cell became. R Chi-square is an estimate of the 'goodness of fit' of the model, and values under 5 indicate a good fit.

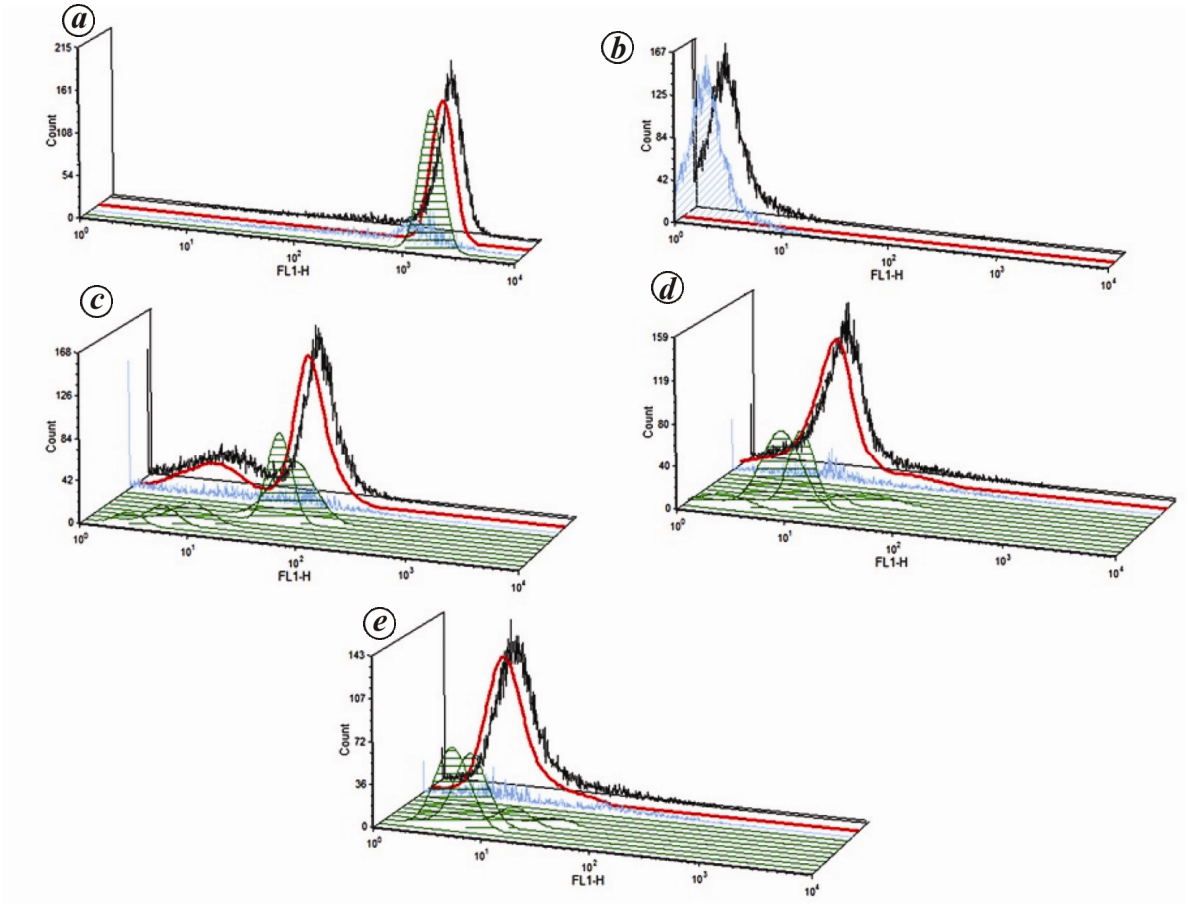

Figure 2. Flow cytometric analyses of K562 cells stained with CFSE before and after the culture period.

monitored at 46.0 peak channel of the FL1 histogram (Figure 2 *c*). For these cells, PI was found as 6.61 (Table 1), and only 13.3% of these cells showed original cell characteristics. The measurement of the fluorescent densities showed that these cells were homogeneous (R Chi-square: 1.35).

Fluorescence intensity was monitored using FL1-H (X-axis); labelling with 'CFSE' represents green flourescein from cells. The black-coloured histogram shows flourescent intensity of untreated CFSE labelled K562 cells before and after 96 h of incubaton. Different doses (0.125–1 mM) of doxorubicine (A), *ortho*- (compound **1**) (B) and *para*- (compound **2**) (C) fluorosubstitute Schiff bases are represented with light and dark gray colours.

Fluorescent intensity of the untreated K562 cells was monitored at 1582.0 peak channel of FL1 histogram immediately after CFSE labelling (Figure 2 *a*). For these cells PI was found as 1.00 (Table 1). Three-quarters of

the cells was found to be undivided, and 72.4% of the cells showed original cell characteristics. The measurement of fluorescent densities showed that these cells were homogeneous (R Chi-square: 1.14). After 96 h of proliferation, fluorescent intensity of these cells was monitored at 1.0 peak channel of FL1 histogram (Table 1, Figure 2 *b*). The measurement of fluorescent densities showed that these cells were nonhomogeneous (R Chi-square: 195.77).

K562 cells labelled with CFSE and analysed using flow cytometry at time 0 h (Figure 2 *a*), cells were re-analysed using flow cytometry at 96 h (Figure 2 *b*) of the culture period. Antiproliferative effects of 1 mM doxorubicine (Figure 2 *c*), 1 mM *ortho*- (compound **1**) (Figure 2 *d*) and 1 mM *para*- (compound **2**) (Figure 2 *e*) fluorosubstitute Schiff bases on CFSE-stained K562 cells at 96 h of the culture period were monitored and identified with coloured lines described as follows. The black line shows the FL1 histogram, which indicates the overall histogram contour, and includes all the data; it does not represent an output of the fitting algorithm. The blue line shows the algorithm determined as noise and is not included into the statistics. The red line shows cell proliferation and indicates the overall contour of the fitted data and excludes the algorithm considered as noise. The green line shows proliferating cells and indicates modelled populations of proliferating cells. These are the actual curves fitted to the data, which represent the parent, undivided population, and all the proliferating generations.

After 96 h of incubation, compounds **1** and **2** treated cells (0.1–1 mM) showed higher level of fluorescence intensity than untreated, but lower level of fluorescence intensity than doxorubicin cells (Figure 1). The intensity of flourescence of the 1 mM compound 1 and 2 treated cells was monitored at 8.82 and 4.66 peak channel of FL1 histogram respectively (Figure 2 *d*, *e*). For these cells the PI was found as 35.29 and 34.30 respectively (Table 1), and only 2.6% of these cells showed original cell characteristics. The measurement of flourescent densities showed that these cells were homogeneous (R Chi-square: 0.94 and 1.08 respectively).

After 96 h of cell culture, doxorubicin was shown to kill the cells strongly with dose dependancy and limit proliferation of the cells, possibly due to high levels of cell death. Cells were shown not to be affected from *ortho*- and *para*-fluorosubstitute Schiff bases as revealed by high viability levels (Figure 3).

In this study we have investigated the antiproliferative properties of doxorubicin, which is currently used for cancer therapy, and fluorinated Schiff bases, which have recently drawn attention for medical use.

Doxorubicin has been used in cancer chemotherapy for many years. It is commonly used in the therapy regimen of some leukemias and Hodgkin's lymphoma, as well as various cancer types[20]. Its interaction with DNA occurs via intercalation and inhibition of macromolecular biosynthesis and thereby it stops the process of replication[21].

In our study, we have shown the antiproliferative potency of doxorubicin on K562 myelomonocytic leukemia cells *in vitro*. We determined the PI of doxorubicin and many other properties such as peak channel, peak value, fitted cells ratio, original cell counts, and undivided cell count, all of which are shown in Table 1, and Figures 1 and 2. Our findings may be a good reference data for researchers who aim to determine the antiproliferative properties of drugs currently used in chemotherapy or anticancer drugs of herbal or chemical sources.

Organofluorine compounds have attracted the interest of researchers because replacement of hydrogen atoms by the highest electronegative fluorine atom(s) or fluorinated groups leads to major changes in lipophilicity, metabolic stability, polarity, reactivity in acidity or basicity, as well as in physicochemical and conformational properties of fluorinated compounds compared with their nonfluorinated analogs[22–24]. The small and highly electronegative fluorine atom is involved in drug–receptor interactions. In addition, the C–F bond of aromatic compounds is metabolically more stable compared with the C–H bond. Their use in different fields such as the chemistry of modern drugs, fluorous biphase chemistry, materials science, and inhibitors, as well as in organic and inorganic synthesis have further simulated interest in the chemistry of fluorinated compounds[13,25,26]. In this study, we have used flourinated (*ortho*- and *para*-monosubstitute) Schiff bases on K562 myelomonocytic leukemic cells to determine their antiproliferative effects. Both *ortho*- and *para*-monosubstituted flour containing Schiff bases possessed antiproliferative effects on K562 cells. The PI for *ortho*-fluorine containing Schiff base (compound **1**) was 35.9;

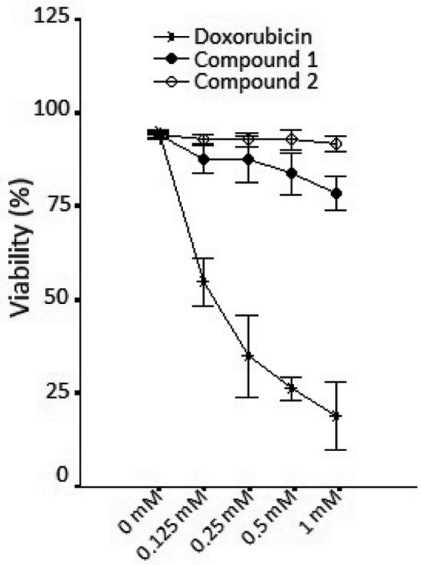

Figure 3. Cell viability of K562 cells cultured with doxorubicin, compounds **1** and **2**.

however, the *para*-fluorosubstitute Schiff base (compound **2**) showed slightly better antiproliferative performance with a PI of 34.6.

We tested doxorubicin, *ortho*- and *para*-fluorosubstitute Schiff base to assess its growth-inhibiting action on K562 cells by trypan blue exclusion. Trypan blue exclusion test is an easy and precise method to test cell viability as well as cell numbers, which can be used to estimate the proliferation of cells as fold increase in comparison with initial cell numbers[27,28]. It is also possible to test the accuracy of CFSE proliferation analysis method. Data from trypan blue exclusion experiments revealed that *ortho*- and *para*-fluorosubstitute Schiff bases are not so effective in killing cancer cells compared to a known anti-cancer drug: doxorubicin.

CFSE is widely used to determine cell proliferation. General protein labelling CFSE acts by forming random covalent bonds with amino groups on cellular proteins, which results in a highly fluorescent membrane impermeable marker[29]. The reduced amount of CFSE due to daughter cells shared the dye, which homogeneously adhered to all compounds of the cells. With each cell division, the dye is divided approximately equally between the two daughter cells[13]. Therefore, fluorescence intensity of the dye is a measure of the number of cells that have undergone divisions. After staining, cell populations are analysed at regular time intervals using flow cytometry and a histogram of the number of cells as a function of dye fluorescence intensity is generated.

Typically, cell proliferation dyes can be used to track up until the fluorescence intensity is reduced to the autofluorescence of unstained cells. In this study, we used a CFSE assay with a different perspective: to determine the antiproliferative effects of doxorubicin and fluorinated Schiff bases in a cancerous cell line. The introduction of fluorescent cell staining and division tracking dyes allowed detection of the amount of cell divisions during proliferation, and to examine the relationship between proliferation and differentiation. Although many immunology studies have been performed using CFSE to determine the proliferation of blood cells[30], here we used CFSE assay as a tool in anticancer research to identify the antiproliferative effects of drugs or chemotherapeutic candidate agents.

Our study may provide a cost effective method for determining the antiproliferative properties of herbal and chemical compounds. Also, obtaining and sharing data about many aspects of antiproliferative properties of anticancer agents will help to quantitively compare data. This new perspective will help researchers to understand how compounds impact on cancer cells.

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Comparative analysis of digestive amylase activity in some tropical and temperate breeds of mulberry silkworm, *Bombyx mori* L.

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In the current study amylase activity was carried out by analysis of digestive fluid in diapausing and non-diapausing strains of mulberry silkworm, *Bombyx mori* L. Six different breeds, viz. Pure Mysore, Nistari, NB_4D_2, SH_6, $SKAUR_6$ and $SKUAST_{28}$ were selected for the study. The average digestive amylase activity was found highest in Nistari during spring (664.82 µg) and summer (993.97 µg) seasons. However, among the bivoltine breeds SKUAST-28 with the average amylase activity during spring (148.47 µg) and summer (144.04 µg) seasons was found to be a superior breed with respect to this parameter. The amylase activity in tropical non-diapausing breeds is higher than that in the bivoltine breeds of silkworm which is responsible for their higher survival rate under unfavourable conditions.

Keywords: Amylase activity, *Bombyx mori* L., diapuasing and non-diapausing strains, maltose, survival.

THE silkworm, *Bombyx mori* L. is an important sericigenous insect which feeds on mulberry leaves and the digestibility of the silkworm larva largely depends on the activity of an enzyme called 'amylase'. Amylase is a hydrolytic enzyme found in micro-organisms, plants and animals which is involved in the digestion and carbohydrate metabolism in insects[1] including carbohydrates available in the form of starch in mulberry leaves[2]. The ability to digest more food influences the growth, development and resistance to diseases, stresses and better survival under different environmental conditions[3]. Amylases from different origins have been characterized[4,5]. The effect of BmNPV infection on the digestive enzyme activity in the silkworm, *Bombyx mori* was studied earlier by others and the activities of amylase, invertase, trehalase and protease were analysed in the infected silkworms[6]. Amylase activity was determined in two Eri silkworm, *Samia cynthia* ricini Boisduval breeds, viz. brick red cocoon and plain white cocoon breeds fed on three different host plants (Castor, *Ricinus communis* L; Tapioca, *Manihot utilessima* Phol. and Barkessuru, *Ailanthus excelsa* Roxb.)[7]. Amylase activity was studied in the digestive juice and haemolymph during the 5th instar larval stage. Comparative studies were carried out on the digestive amylase activity of 4th and 5th instar larvae of *Antheraea mylitta* Drury during outdoor and indoor rearing programmes[8].

Keeping in view the role of amylases in better digestibility and consequently the survival of silkworms, the present study was carried out to evaluate the difference in the amylase activities of diapausing and non-diapausing strains of silkworm, *Bombyx mori* L.

Six breeds of the mulberry silkworm comprising two multivoltines (Pure Mysore and Nistari), two temperate bivoltine breeds (SKAU-R-6 and SKUAST-28) and two tropical bivoltine breeds (NB_4D_2 and SH_6) were used in the present study. Disease-free layings of these races were obtained from the germplasm bank maintained at the Central Sericultural Germplasm Resource Centre (CSGRC), Hosur (Tamil Nadu) and the Silkworm Breeding and Genetics section TSRI (SKUAST-K), Mirgund.

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