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The application of pig blood in the *in vitro* measurement of platelet adhesion

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Platelet adhesion to the damaged blood vessel is an essential step in the process of local hemostasis regulation. Different pharmaceuticals alter platelet susceptibility to physiological stimulation, therefore it is necessary to determine drug influence on platelet reactivity [1].
A method of measurement of human blood platelet adhesion to protein coated multiwell microplates has been previously agonists described by Bellavite et al. [2]. It is a simple and reproducible cell adhesion assay based on the determination of the acid phosphatase (PA) activity of platelets. As human blood is a precious material of restricted availability for research purposes, in this report the colorimetric assay of microtiter plate adhesion was applied using pig blood. Pig blood is easily available in substantial quantities, and its properties, as well as the similarities between human and pig morphological elements, enable the application of pig blood in *in vitro* assays [3, 4].
Our data demonstrated that the type of coating proteins used determined percentage of adhesion. The most sensitive stimulation of pig platelets with human Fb (Kabi) was observed. The results of PA activity in the case of both types of platelets (human and pig) are close, therefore the colorimetric assays of adhesion may be used in *in vitro* studies with platelets isolated from pig blood. In our opinion the assay described may be applied for screening purposes of new drugs.

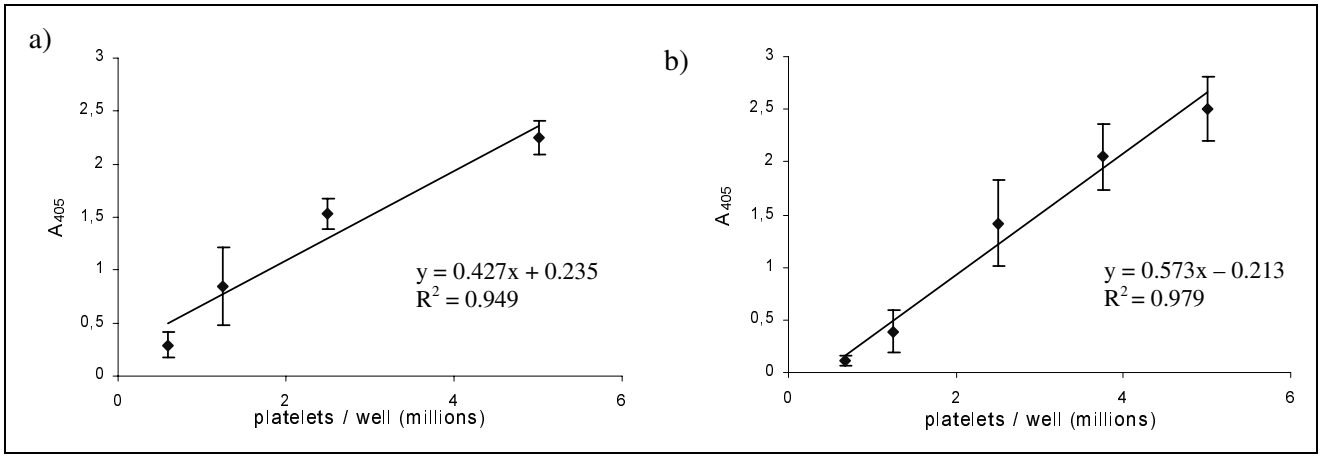

Fig.: Acid phosphatase activity as a function of a) pig and b) human platelet numbers (60 min incubation). Values are means ± SD (n = 3–11)

Table: Pig blood platelet adhesion to various proteins

	% Adhesion ± SD			
	Fibrinogen		Collagen	
	Human (Kabi)	Bovine (Serva)	Calf skin (Sigma)	Type I (Chrono – log)
Without ADP	15.4 ± 4.6 (n = 15)	17.1 ± 4.9 (n = 16)	16.9 ± 8.5 (n = 12)	16.0 ± 2.6 (n = 12)
With 10 µmol/l ADP (Sigma)	27.2 ± 4.6 (n = 15)	20.0 ± 5.2 (n = 20)	16.7 ± 8.3 (n = 11)	15.3 ± 2.2 (n = 12)

Experimental

The assays with pig blood collected into ACD (15 g/l citric acid, 20 g/l glucose, 25 g/l sodium citrate) in 5 : 1 proportion, were performed according to the method of Bellavite et al. [2], with minor modifications. For all measurements the platelet suspension in buffer A (pH 7.4; 145 mmol/l NaCl; 5 mmol/l KCl; 10 mmol/l HEPES; 0.5 mmol/l Na_2HPO_4; 6 mmol/l glucose; 0.2% bovine serum albumin) was used. Pig blood (about 1000 ml) was initially centrifuged at 352 × g, 20 °C for 30 min; the separated plasma was centrifuged again at 285 × g, 20 °C for 20 min. Platelet rich plasma (PRP) was centrifuged at 529 × g, 20 °C, 15 min and the platelet's bottom was suspended in 15 ml of buffer A and centrifuged at 529 × g, 20 °C, for 15 min. The remaining platelets were finally resuspended in 3 ml of buffer A.

Comparative assays with human blood obtained from healthy volunteers were also performed. A final volume of 7 ml of human blood was drawn by venipuncture in 1.2 ml of ACD. Blood was centrifuged at 300 × g, 20 °C, for 10 min, next PRP was centrifuged 700 × g for 15 min and platelet's bottom were suspended in a buffer A.

Suspensions of pig and human platelets were incubated for 10 min. in a water bath at 37 °C, prior to assay. Then, it was diluted with buffer A to obtain the desired platelet cell count (5.6×10^7/ml). The platelet count was determined with a spectrophotometric assay [5].

Microtiter plates (NUNC, U.S.A.) were coated with 200 µl human (Kabi, Sweden) or bovine (Serva, Germany) fibrinogen (Fb) solution at the concentration of 2 mg/ml PBS (pH 7.40; 20 g/l KCl, 0.20 g/l KH_2PO_4, 0.047 g/l $MgCl_2$, 8.00 g/l NaCl, 1.150 g/l Na_2HPO_4) or with a collagen (Sigma – Aldrich, Chrono-log, USA) solution (20 µg/ml in 0.9% NaCl). The closely sealed plate was incubated overnight at 4 °C. After coating, the plate was washed with 0.9% NaCl, and then 25 µl of buffer A, containing 4 mmol $CaCl_2$ and 4 mmol $MgSO_4$, was added into each well. Following the plate preparation, as described above, 45 µl of platelet suspension (2.5×10^6/well) was added into the plates. In some cases, 10 µl ADP (Sigma – Aldrich) (1mmol/l) was added to stimulate adhesion. The plate was then incubated in a water bath for 60 min at 37 °C. At the end of incubation, nonadherent platelets were washed out manually with PBS at room temperature. Further, a citrate buffer, 150 µl (pH 5.4) with 5 mmol/l p-nitrophenyl phosphate and 0,1% Triton X-100 (that causes platelet lysis), was added into each microtiter plate. The plate was incubated for 60 min at room temperature. Then, a 100 µl NaOH solution (2 mol/l) was added to terminate the reaction and develop the color. As a product of PA reaction, p-nitrophenol was generated, and its concentration was assayed colorimetrically with a microplate reader (ELx 800; Bio-Tek Instruments) at 405 nm against a platelet free blank. The percentage of adherent cells was calculated on the basis of a standard curve obtained with a know number of platelets (Fig.).

The measured acid phosphatase activity was very close for both types of platelets. PA activity in pig platelets was 0.655 ± 0.145 absorbance units/60 min/10^6 platelets ($n = 8$, $min = 0.510$; $max = 0.800$; $W = (SD/\overline{X}*100) = 22.1\%$) and the respective value in human blood was 0.598 ± 0.13 ($n = 11$; $min = 0.374$; $max = 0.791$; $W = 21{,}1\%$).

Human blood assayed with Bellavite et al. [2] method was characterized with the following values 0.589 ± 0.12 ($n = 32$; $min = 0.380$; $max = 0.823$; $W = 20\%$); values of % adhesion/60 min of incubation ± SD: 4.9 ± 2.6 and, after the activation with ADP (10 µmol/l) 20.7 ± 2.6 for human Fb (type I; Sigma) and 27.4 ± 3.4 and, with ADP 29.6 ± 3.8 for collagen (Menarini, Italy) adhesion.

In our studies with human platelets percentage of adhesion to human Fb (Kabi) was 3.36 ± 3.01 and, after the activation with ADP (10 µmol/l) 15.25 ± 5.75; $n = 9$. The higher percentage of adhesion was found for pig platelets (Table 1) with the use of human Fb (especially after activation with ADP) and with bovine Fb, as compared with our results obtained for human platelets. In the presence of the collagen types applied, adhesion did not increase further after ADP stimulation (Table). Similar result adhesion to collagen was observed in investigation with human blood [2].

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Cytotoxic alkaloids from *Tylophora indica*

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The leaves of *Tylophora indica* (Burm. F.) Merr. (Asclepiadaceae) are emetic, diaphoretic, expectorant [1]. The roots possess stimulant, emetic, cathartic, expectorant, stomachic and diaphoratic properties and are used to treat asthma, bronchitis, whooping cough, dysentery, and diarrhoea [2]. The plant is an indigenous substitute for ipecacuanha [1] and its alkaloids show anti amoebic activity [3].

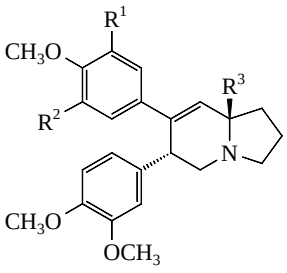
The phenanthroindolizidine alkaloids tylophorine [4], septicine [4], tyloindicines A-J [5, 6], 14-hydroxy-isotylocrebrine [5], 4,6-desdimethylisotylocrebrine [5], and tyloindane [6], have been reported as constituents.

An anti-tumor activity screening study of tyloindicines F(**1**), G(**2**), H(**3**), I(**4**) [6] was carried out following the National Cancer Institute (NCI) *in vitro* programme based on the use of a panel of 58 cell lines of major human tumors derived from nine cancer types including leukaemia, melanoma, lung, colon, kidney, CNS, ovary, prostate and breast tumors [7, 8]. Current investigational approaches to data analysis and interpretation have been provided elsewhere [9, 11].

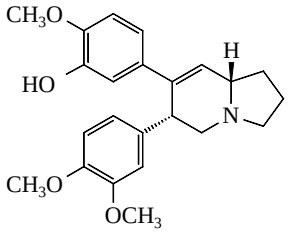
The bioassay was carried out according to procedures described in the NCI protocols for *in vitro* human disease oriented preliminary screening of drugs and crude extracts for anti tumor activities [12, 13]. The alkaloids were tested at five concentrations at tenfold dilution as reported earlier [8]. The data are presented as horizontal bars extending either to the right or left of the mean depending on the sensitiveness of the cell lines (Figs. 1, 2) [14]. These lines were provided at the percentage growth value of +50, 0 and −50. The length of each bar is proportional to the relative sensitiveness of the cell line. Thus, each compound can be represented by a characteristic "finger print" of cellular responsiveness.

In the *in vitro* primary disease oriented anti-tumor screen, compounds **1**–**4** exhibited up to a 1000 fold range of differential sensitivity as shown in Figs. 1 and 2. At concentrations of 10^{-5}–10^{-8} M, the compounds typically produce LC_{50}-level responses against the majority of the cell lung cancer and melanoma cell lines and some of the CNS cancer lines.

Phenanthroindolizidine alkaloids show an interesting profile of cytotoxic activity. To our knowledge, this is the first report of secondary metabolites from *Tylophora* species having such an activity.



Tyloindicine F. R^1=R^2=H, R^3=OH
Tyloindicine G. R^1=OCH_3, R^2=H, R^3=OH
Tyloindicine I. R^1=OCH_3, R^2=OH, R^3=H



Tyloindicine H