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Screening for the P-Glycoprotein Inhibitory Pump Activity of Plant Extracts that are Used in Tanzanian Traditional Medicine

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An *in vitro* screening experiment of 45 methanolic plant extracts was carried out to investigate the inhibition of P-glycoprotein (P-gp) in MCF-7R cells. The investigation was carried out using standard functional assay with rhodamine 6G as the fluorescent probe and reserpine, a known inhibitor of P-glycoprotein pump, was used as a reference drug. The results revealed that out of the 45 plant extracts tested, 3 extracts i.e. *Bauhinia thoningii*, *Clerodendrum myrico*ides and *Rhus natalensis* exhibited pronounced activity at the concentration of 100 μg/ml. In comparison to the negative control, *B. thoningii*, *C. myrico*ides and *R. natalensis* extracts inhibited the pump by a factor of about 2.5, 4 and 4.3, respectively. The remaining 42 extracts did not show any activity. The reference drug reserpine (20 μM) inhibited the pump by a factor of about 11. These preliminary results indicate that the inhibition of P-gp in MCF-7R cells is through a concerted action of compounds present in the extracts and that if isolated might be effective P-gp pump inhibitors in the treatment of cancer.

Keywords: Plant extract; P-glycoprotein inhibition; rhodamine 6G; MCF-7R cells.

INTRODUCTION

Simultaneous resistance of tumour cells to multiple cytotoxic drugs as well as of bacteria to antibacterial agents is a phenomenon known as multidrug resistance (MDR). This resistance, which may be intrinsic or acquired, is a major limitation to the successful chemotherapeutic treatment of cancer and infections [1].

An important goal of cancer research has been to find ways to overcome or circumvent this drug resistance. However, it has been difficult to study drug resistance *in vivo,* mainly due to heterogeneity of human cancer or difficulty in obtaining several clinical specimens from the same tumour during the course of treatment. Consequently, a large number of *in vitro* models, which can be used to study drug resistance in cancer cells, have been developed from different species.

Studies on the molecular basis of MDR have revealed that multidrug-resistant cells differ from their drug-sensitive parental cells by: (i) increased drug efflux (ii) decreased drug sensitivity (iii) increased DNA repair mechanisms and (iv) altered expression of metabolic and detoxification processes. It is well established that the major mechanism of MDR in human cells involves the expression of a 170 kDa plasma membrane P-glycoprotein (P-gp) encoded by the *mdr1* gene [2]. P-gp belongs to the superfamily of transporter proteins containing an ATP-binding cassette [3]. It is thought to function as a broad-substrate ATP-dependent pump, which exports drugs out of mammalian cells, thus lowering the intracellular drug concentration below the cytotoxic threshold [4].

As an *in vitro* model to study MDR, drug sensitive culture cells are selected stepwise by increasing concentrations of a cytotoxic agent (e.g. doxorubicin and vincristine) thereby surviving cells acquiring a phenotype of cross-resistance to a variety of structurally and functionally unrelated anticancer drugs (e.g. vinca alkaloids, anthracyclines and taxanes). Using such an *in vitro* model, the following parameters can be used to screen agents that overcome MDR: (i) enhanced cytotoxity of anticancer drug to MDR cells (ii) enhanced accumulation of anticancer drugs in MDR cells (iii) enhanced accumulation of fluorescent basic dyes (e.g. rhodamines) that are also candidates of the P-gp pump in MDR cells [5].

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One possible approach to reverse or circumvent drug resistance is through chemosensitizing agents that are relatively not toxic themselves but enhance the cytotoxic effects of chemotherapeutic agents. It is known that many agents, including the calcium-channel blocker, verapamil, the antihypertensive agent, reserpine, the immunosuppresive agent cyclosporin A and the estrogen antagonist tamoxifen can interfere with the function of P-gp and therefore circumvent MDR [6-8]. However, these drugs have their own clinical limitations including toxicity as well as low specificity. In this regard, there is therefore a need for discovery of effective and safe MDR chemosensitizers.

The objective of the present work was to screen for the MDR reversal activities of plant extracts. Some of the extracts used in this study are widely reported to prevent and treat a number of diseases in certain traditional communities [9,10]. A few of these claims have been authenticated by scientific biomedical investigations [11]. However, nothing is known about their potential as P-gp inhibitors. The ultimate goal of this work was therefore to screen for the P-gp inhibitory activity of the plant extracts. The results obtained from this study might lead to the isolation of active MDR chemosensitizers from the active plants extracts.

MATERIALS AND METHODS

The MCF-7R cell line, culture medium and solvents MCF-7R (human breast adenocarcinoma resistant to doxorubicin) cells were a generous gift from Dr. J. Carmichael (University of Nottingham, UK) and were originally derived in the laboratory of Dr. Kenneth Cowan (National Cancer Institute, MD, USA). Cells were maintained at 37 °C in a humidified atmospheric air containing 5 % CO_2, in nutrient mixture (F10/HAM) containing L-glutamine, with fetal calf serum (10 %), non-essential amino acids (1 %), tylosin (60 µg/ml) and antibiotic/antimycotic solution (1 % v/v). Nutrient mixture, fetal calf serum, non-essential amino acids, penicillin, streptomycin, amphotericin B and phosphate-buffered saline (PBS) were obtained from Gibco BRL (UK). Tylosin was obtained from Eli Lilly (Belgium) and sodium dodecyl sulphate (SDS), was purchased from Fluka Biomedika (Germany).

Validation of MCF-7R cells for the expression of P-gp pump

MCF-7 is a human breast adenocarcinoma cell line and it was first obtained from the pleural effusion of a female cancer patient.
MCF-7 resistant (MCF-7R) cells may be obtained by incubating the parent non-resistant cells with sub-lethal doses of a chemotherapeutic agent, such as doxorubicin, which would cause the induction of P-gp expression. The resistant sublines are isolated and re-incubated with higher concentrations of the chemotherapeutic agent. This is repeated several times to ensure that the final culture obtained contains cells that are resistant to the drug. Using cDNA microarray technique several genes have been identified in MCF-7R cells whose expression is induced in response to doxorubicin treatment [12].

Methods used for quantitative determination of modulation of expression of drug transport mechanisms include staining cancer specific antibodies in Western blotting, immuno-precipitation and/or radio-labeling with specific drugs, flow cytometry and microscopy assays. In this work, tests were carried out to validate the presence of plasma membrane drug transporter proteins in a cell culture of MCF-7R *in vitro* using a standard functional assay with rhodamine 6G as the fluorescent probe. Reserpine, which is a known inhibitor of membrane transporter proteins [8], was used in different concentrations to assess its effectiveness in reversing rhodamine 6G extrusion from the MCF-7R cells.

Both MCF-7R and MCF-7 parent cells were seeded on a 96-well tissue microtitre culture plate (Falcon, NJ, U.S.A.) at 3 x 10^4 cells in 200 µl of nutrient mixture per well. The plates were incubated at 37 °C for 24 h in a humidified atmospheric air containing 5 % CO_2. Afterwards, the culture medium was discarded and the cells were incubated with medium containing 0.3 µM of rhodamine 6G (Sigma, MO, USA) together with different concentrations (0, 1, 2, 5, 10, 20 and 50 µM) of reserpine (Sigma, MO, USA) for 3 h.

Rhodamine 6G, a candidate for P-gp pump was used as a fluorescent probe. The contents were discarded and the cells were washed twice with 200 µl cold-ice PBS. They were trypsinised with

100 µl phenol-red free trypsin solution for 15 minutes and the trypsin-cell suspension was transferred into the wells of a new 96-well plate. In order to solubilise the cells and release rhodamine 6G, 100 µl of 4 % SDS solution in PBS was added to each well and the plates were shaken continuously for 2 h.

The cellular accumulation of rhodamine 6G was detected by measuring the fluorescence of the dye released after solubilisation using microplate fluorescence reader (FL 600 Bio-tek Winooski, USA) at excitation and emission wavelengths of 530 and 590 nm respectively. The experiment was carried out 6 times and the results were expressed as the mean fluorescence units in the 6 tests.

Plant material

All plant materials were collected from different parts of Tanzania and their respective voucher specimens are deposited at the Department of Pharmacognosy, School of Pharmacy of the Muhimbili University College of Health Sciences, Dar es Salaam, Tanzania. Plant materials were dried at room temperature and ground. Methanolic crude extracts were obtained from the dry powder by maceration. Dry plant extracts were then obtained by evaporation of the solvent under reduced pressure.

P-gp-mediated drug efflux inhibition assay of plant extracts

Forty-five methanolic plant extracts (listed in table 1) were screened for the inhibition of P-gp-mediated drug efflux. Stock solutions of extracts were prepared by dissolving dry methanolic extracts in dimethyl sulphoxide (DMSO) to give 100 mg/ml and 10 mg/ml concentrations. Immediately before incubation with the cells, DMSO extract solutions were diluted x1000 with culture medium to give the 100 µg/ml and 10 µg/ml concentrations.

MCF-7R cells were seeded in the wells of 96-well tissue microtitre culture plates at a density of 3 x 10^3 cells in 200 µl of medium per well. The plates were incubated at 37 °C for 24 h in a humidified atmospheric air containing 5 % CO_2. The medium was replaced with fresh medium containing 0.3 µM of rhodamine 6G together with 10 µg/ml or 100 µg/ml concentrations of each extract or 20 µM of reserpine. The plates were further incubated for 3 h at 37 °C. Afterwards, the cells were washed twice with 200 µl cold-ice PBS and trypsinised (with 100 µl phenol-red free trypsin solution) for 15 minutes. The trypsin-cell suspension was transferred into the wells of a new 96-well plate. SDS (in PBS) was added to each well and the plates were shaken for 2 h. The cellular accumulation of rhodamine 6G released after solubilisation was determined by measuring the fluorescence of the dye using microplate fluorescence reader as described above. The experiment was carried out 6 times and the results were expressed as the mean fluorescence units in the 6 tests.

RESULTS AND DISCUSSION

Using MCF-7R cells, an *in vitro* bioassay-targeted process was carried out to identify the extracts which could inhibit the P-gp mediated efflux of rhodamine 6G from MCF-7R cells. The fluorescence of rhodamine 6G measured from the cells which had been incubated with medium containing the extracts was compared with the fluorescence of the dye measured from the cells that were incubated with rhodamine 6G alone (negative control).

Reserpine, a known natural inhibitor of the P-gp pump [8] was used in this study as a reference drug, as well as for validation of the P-gp expression in MCF-7R cells. The results show that there was low fluorescence of rhodamine 6G recovered from the cells which had been incubated with medium without reserpine (negative control). In comparison, the parent-sensitive cell line MCF-7 gave higher fluorescence of about a 12.5-fold (figure 1). There was however a significant and steady increase in the uptake of rhodamine 6G by the MCF-7R cells in the presence of increasing concentrations of reserpine. The highest concentration of 50 µM gave a maximum inhibitory effect, which led to a 11.9-fold cellular accumulation of rhodamine 6G in comparison with the negative control (0 µM reserpine).

On the other hand, there was no remarkable increase in uptake of rhodamine 6G by the MCF-7 parent cells, in the presence of various concentrations of reserpine (Figure 1).

Table 1: List of tested plant species with their respective part used

	Plant species (Family)	Part used
1.	*Ziziphus mucronata* Willd. (Rhamnaceae)	Root
2.	*Lannea stuhlmanni* Engel. (Anacardiaceae)	Root
3.	*Acacia polyacantha* Willd. (Mimosaceae)	Root
4.	*Acacia nilotica* (L.) Del. (Mimosaceae)	Root
5.	*Oxygonium sinuatum* (Meisn.) Dammer (Polygonaceae)	Whole plant
6.	*Dombeya shupange* Sprague (Sterculiaceae)	Root bark
7.	*Parinari excelsa* Sabine (Chrysobalanaceae)	Stem bark
8.	*Ximenia caffra* Sond. (Olacaceae)	Root
9.	*Salacia madagascariensis* (Lam.) DC. (Celastraceae)	Root
10.	*Cassia abbreviata* (L.) Det. (Mimosaceae)	Root
11.	*Dichrostachys cinerea* (L.) Wight and Arn. (Mimosaceae)	Whole plant
12.	*Pterocarpus angolensis* DC (Papilionaceae)	Root
13.	*Margaritaria discoidea* (Baill.) Webster (Euphorbiaceae)	Root bark
14.	*Rhus natalensis* Krauss (Anacardiaceae)	Root
15.	*Vangueria tomentosa* Hochst. (Rubiaceae)	Root
16.	*Markhamia obtusifolia* (Bak.) Sprague (Bignoniaceae)	Root
17.	*Opilia celtidifolia* Guill. And Perr. (Opiliaceae)	Stem
18.	*Uvaria acuminata* Oliv. (Annonaceae)	Root
19.	*Uvaria leptocladon* Oliv. (Annonaceae)	Root
20.	*Bauhinia thoningii* Schumach. (Leguminoceae)	Stem bark
21.	*Keetia zanzibarica* (Klotsch) Bridson (Theophrastaceae)	Root
22.	*Entanda abyssinica* A. Rich. (Mimosaceae)	Root bark
23.	*Octea usambarensis* Engl. (Lauraceae)	Root bark
24.	*Zanthoxylum chalybeum* Engl. (Rubiaceae)	Root
25.	*Minopsis fraticosa* Boj. (Sapotaceae)	Root
26.	*Pteleopsis myrtifolia* (Laws.) Engl. And Diels (Combretaceae)	Root
27.	*Kigelia africana* (Lam.) Benth. (Leguminoceae)	Stem bark
28.	*Clerodendrum myricoides* (Hochst.) (Verbenaceae)	Root bark
29.	*Strychnos innocua* Del. (Loganiaceae)	Root
30.	*Harunguna madagascariensis* Poir. (Guttiferae)	Leaves
31.	*Canna didymobotrya* Fres. (Leguminoceae)	Leaves
32.	*Canavaria rosea* DC. (Papilonaceae)	Seeds
33.	*Fluggea virosa* Baill. (Euphorbiaceae)	Root bark
34.	*Thylachium africanum* Lour (Capparidaceae)	Leaves
35.	*Gnidia krausiana* Meisn. (Thymelaceae)	Root
36.	*Harissonia abyssinia* Oliv. (Simaroubaceae)	Root
37.	*Crinum papillosum* Nordal (Amarilidácea)	Whole plant
38.	*Gloriosa superba* L. (Liliaceae)	Root
39.	*Markhamia obtusifolia* (Bak.) Sprague (Bignoniaceae)	Root
40.	*Paulinia pinnata* L. (Sapindaceae)	Aerial parts
41.	*Bridelia micrantha* (Hochst.) Baill. (Euphorbiaceae)	Root bark
42.	*Capparis sepiaris* L. (Capparidaceae)	Root bark
43.	*Hymenocardia mollis* Pax (Euphorbiaceae)	Root bark
44	*Euphorbia quandrangularis* Pax (Euphorbiaceae)	Root
45.	*Uvaria lucida* Benth. (Anonaceae)	Stem bark

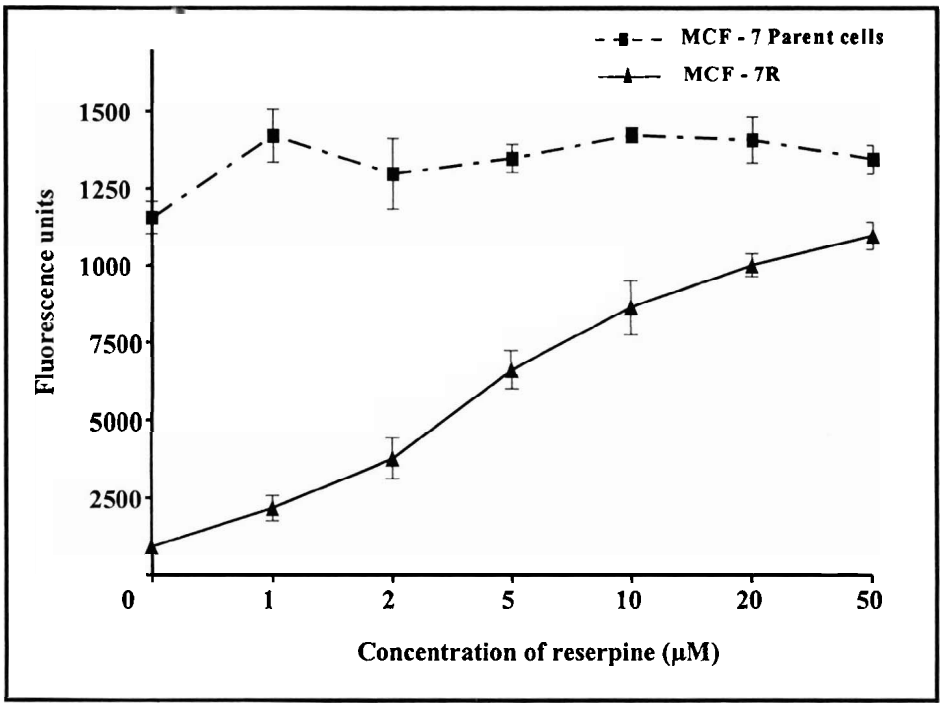

Figure 1: Fluorescence intensities of rhodamine 6G recovered from MCF-7 parent and resistant (MCF-7R) cells in the presence of increasing concentrations of reserpine (mean ± SD, n = 6).

MCF cells only showed a 1.2-fold increase in fluorescence with 50 μM concentration of reserpine in comparison with the negative control. Taken together, these results show that MCF-7R cells used in this study express the P-gp pump which pumps out the rhodamine 6G dye from the cells.

Studies have shown that rhodamine 6G is taken up by the cells intracellularly and selectively stains mitochondria at low concentrations, and endoplasmic reticulum at high concentrations [13]. In addition, quantitative and qualitative studies have demonstrated that MCF-7R cells express P-gp pump in their membranes [14].

This means that an extract or compound which inhibits the dye efflux from the *mdr1* gene-expressing MCF-7R cells is a modulator of the drug pump, and should act by inhibiting the activities of membrane transporter proteins that pump drugs out of the cells.

Results of the present study show that out of the 45 plant extracts tested, 3 extracts had an inhibitory effect on the pump. In comparison to the control (in which the cells were only incubated with rhodamine alone), *Bauhinia thoningii*, *Clerodendrum myricoides* and *Rhus natalensis* extracts inhibited the dye efflux by a factor of about 2.5, 4 and 4.3, respectively (Figure 2).

The activity of these extracts was demonstrated at the concentration of 100 μg/ml, whereas at 10 μg/ml concentration the extracts were ineffective. The results of the remaining 42 extracts are not shown but they were similar to that of the negative control, an indication that these extracts did not have any affect on the pump at both concentrations tested. Since it has been shown that rhodamine compounds are transported by P-gp [15,16], the results of this study support the premise that the active compounds in the active extracts reverse drug efflux in MCF-7R cells by inhibiting the P-gp pump.

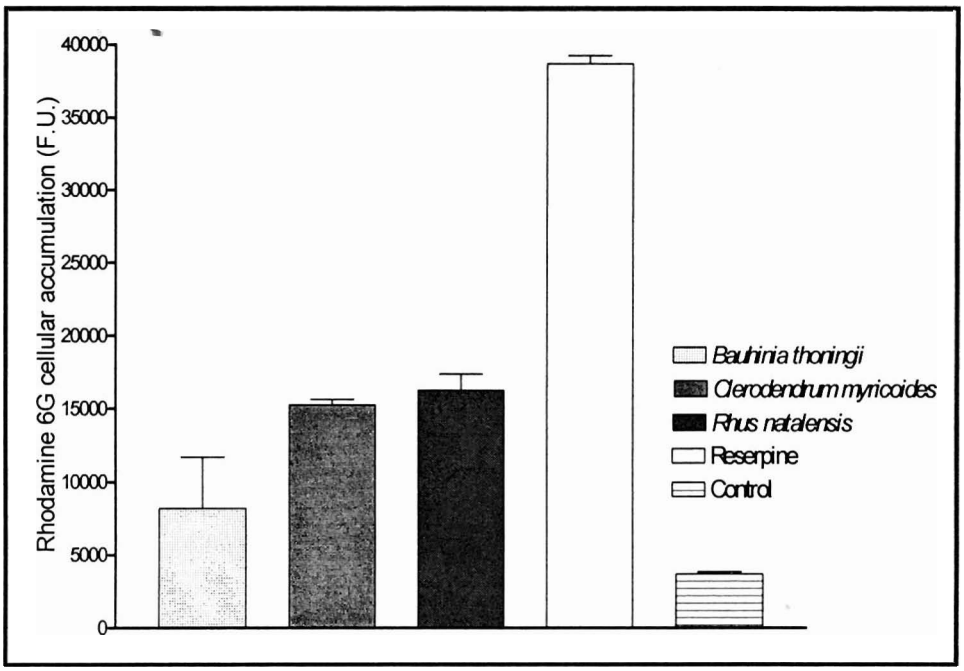

Figure 2: Results of the three active extracts at 100 µg/ml, the positive control (20 µM reserpine) and a negative control (mean ± SD, n = 6)

Besides, some work was done to find out whether the extracts on their own could contribute to the fluorescence observed after exposure of the cells to rhodamine 6G. This could be possible if the compounds in the fractions fluoresce at excitation and emission wavelengths of 530/25 and 590/35 nm, respectively. It was observed that there was no intracellular fluorescence induced by any extract used in this study. This indicates that the extracts did not fluoresce on their own and that the fluorescence was solely due to rhodamine 6G accumulation in the cells.

It should however be mentioned that the lack of activity from the 42 extracts tested in this study does not necessarily mean that the extracts do not contain MDR-reversing agents. This is due to the fact that the bioassay model used in the present study was based only on P-gp-mediated MDR. Although it is acknowledged that this is the main mechanism responsible for extrusion of chemotherapeutic agents from cancer cells, other mechanisms of MDR apart from P-gp pump do exist [17].

Taken together, the results of the present study show that out of the 45 methanolic plant extracts tested, three extracts (*B. thoningii*, *C. myricoides* and *R. natalensis*) exhibited pronounced inhibition of P-gp-mediated rhodamine 6G efflux from MCF-7R cells. This is an indication that the extracts are likely to contain compounds that are potential inhibitors of P-gp-mediated cellular drug efflux. More experiments are needed to isolate and purify the active compounds from these plant extracts, and to test whether these compounds can inhibit the efflux of chemotherapeutic agents from cancer cells, thereby restoring the sensitivity of the otherwise drug-resistant cancer cells.

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