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ScienceDirect
Methods 42 (2007) 377–387
METHODS
www.elsevier.com/locate/ymeth

The sulphorhodamine (SRB) assay and other approaches to testing plant extracts and derived compounds for activities related to reputed anticancer activity

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Accepted 10 January 2007

Abstract

Since the major approach in searching for potential anticancer agents over the last 50 years has been based on selective cytotoxic effects on mammalian cancer cell lines, cell-based methods for cytotoxicity are described and compared. The sulphorhodamine B (SRB) assay is described in detail as the preferred method and also a novel approach has been developed which is based on the hypothesis that, in some circumstances, the naturally occurring compounds act as prodrugs rather than active compounds in their own right. Consequently, extracts or compounds are pre-incubated with systems modelling metabolic processes in the body before being tested. The methods have been validated using known compounds and *Iris tectorum* extracts have been shown to be more cytotoxic after treatment with β-glucosidase.

In addition bioassays based on mammalian cells involving antioxidant and upregulation of some cellular self-defence mechanisms are discussed which are related to prevention as well as treatment of cancer. Extracts of *Alpinia officinarum* induced glutathione-*S*-transferase (GST) activity in cultured hepatocytes and this was traced to the phenylpropanoids present, especially 1′-acetoxychavicol acetate.

Keywords: Cytotoxicity testing; SRB assay; Antioxidant; GST upregulation; Phenylpropanoids; Flavonoids; Alpinia

1. Introduction

A significant part of drug discovery in the last forty years has been focussed on agents to prevent or treat cancer. This is not surprising because, in most developed countries and, to an increasing extent, in developing countries, cancer is amongst the three most common causes of death and morbidity. Treatments for cancer may involve surgery, radiotherapy and chemotherapy and often a combination of two or all three is employed.

Natural compounds from flowering plants play a significant role in cancer chemotherapy. Anticancer drugs in wide clinical use include vincristine and vinblastine from *Catharanthus roseus*, palitaxel (Taxol®) and taxotere from species of yew (*Taxus*), etoposide derived from lignans of *Podophyllum* spp. and camptothecin analogues, such as topotecan, from *Camptotheca acuminata.* All of these are fundamentally cytotoxic and act principally by inhibiting cell proliferation, but by different mechanisms. In fact, some natural products have been found to act by novel mechanisms and so have enabled novel targets to be developed for screening, exemplified by the discovery that paclitaxel inhibited mitosis by stabilising microtubules and so preventing their depolymerisation back to tubulin, in contrast to many other anticancer agents which inhibit the formation of microtubules in the first place [1].

In spite of these successes, there is still much activity directed to finding novel anticancer agents. The traditional cytotoxic approach is associated with severe and unpleas-

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doi:10.1016/j.ymeth.2007.01.003

ant side effects in clinical usage so a 'cocktail' of lower doses of such compounds is now often given, rather than a large, and therefore more toxic, dose of a single compound. It is relatively easy to screen extracts and compounds for cytotoxic effects and large throughput automated screening procedures are used in industry and by research organisations such as the National Cancer Institute in USA, which employs 60 different cancer cell lines. Common methods for estimating cytotoxic activity are discussed below, but it should be noted that other types of experiments have to be conducted to deduce the mechanisms responsible and whether cell death is due to necrosis or apoptosis. These are beyond the scope of this paper.

In the last twenty years, interest has grown in the links between dietary and environmental factors and the incidence of various cancers. There is increasing evidence that some constituents of plants found in the diet prevent, at least to some extent, the damage to the cell or other factors in its metabolism and function, which pre-dispose people to cancer. Testing for such preventive activities is not so common as cytotoxicity testing but some approaches that have been tried are discussed below.

2. Cytotoxicity testing

Cytotoxicity testing is based on one or more mammalian cell lines being grown under conditions where they are actively growing and undergoing mitotic division. Cells are cultured in a microtitre well plate and the rate of multiplication and growth is measured indirectly by formation of a colour, the intensity of which is directly proportional to the number of cells present. A variety of experiments can be used and the most basic is to compare the rate of proliferation of a cancer cell line in the presence and absence of the test substance, usually after a specified time. Ideally several different cancer cell lines can be used so that selectivity can be assessed and the addition of normal cell lines to the battery enables selectivity between cancer cell lines and normal cell lines to be determined. This gives an indication of potential usefulness in a clinical setting, for which a selectivity of at least two orders of magnitude in favour of the cancer cell line being the more susceptible is required [2].

Such tests can also be used to determine whether the cytotoxic effect is merely cytostatic i.e. it stops cells growing or dividing, or cytocidal, where the cells are killed. For such a determination, two sets of identical cells are both exposed to the test agent under identical conditions and for the same period of time. At the end of the exposure period, one set of cells is assayed whilst, for the other set, the medium containing the test substance is discarded and replaced by fresh medium alone. The cells are then incubated for a fixed time before the assay for cell growth is conducted. If the agent has only a cytostatic effect, the cells will grow and undergo mitosis in the fresh medium but, if they have been killed during the initial exposure time, no such increase in number of cells will be observed.

Two major techniques are used to assess the cell growth. The first one uses either 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2*H*-tetrazolium-5-carboxanilide sodium salt (XTT). The MTT method was the first developed and was introduced in 1986 [3] followed by the use of XTT in 1988 [4]. Both of these reagents are metabolically reduced by the mitochondria in viable cells to a coloured formazan product, the intensity of which can be measured spectrophotometrically in a plate reader. The use of XTT is preferred since the formazan produced is soluble in water and the solubilisation step required if MTT is used is eliminated [4]. However many cell lines were not so efficient at reducing XTT compared with MTT but the addition of phenazine methosulphate (PMS) showed that reduction was much better [4].

With both of these reagents the formation of colour relies on the activity of the mitochondria so, if the function of these is inhibited by variations in cellular levels of NADH, glucose and other factors, variable results are obtained and a similar result may be given as if the cells were not alive or not proliferating.

Because of these limitations, the second major technique for testing cytotoxicity is the more preferred i.e. the sulphorhodamine B (SRB) assay. This relies on the uptake of the negatively charged pink aminoxanthine dye, sulphorhodamine B (SRB) by basic amino acids in the cells. The greater the number of cells, the greater amount of dye is taken up and, after fixing, when the cells are lysed, the released dye will give a more intense colour and greater absorbance [5]. The SRB assay is sensitive, simple, reproducible and more rapid than the formazan-based assays and gives better linearity, a good signal-to-noise ratio and has a stable end-point that does not require a time-sensitive measurement, as do the MTT or XTT assays [6,7].

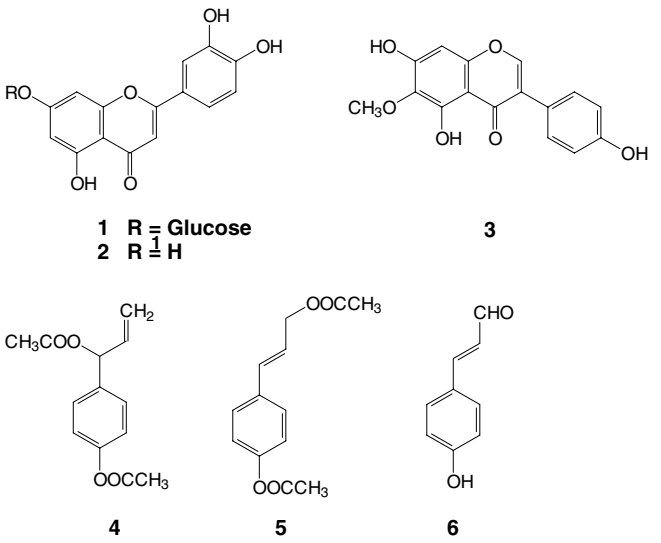

Fig. 1. Structures of compounds. **1**, Luteolin-7-*O*-glucoside; **2**, luteolin; **3**, tectorigenin; **4**, trans-*p*-coumaryl diacetate; **5**, 1′-acetoxychavicol acetate; **6**, 4-hydroxycinamaldehyde.

Both the formazan-based and SRB assays have been used extensively, the latter being that used in the NCI screen and the preferred method in our laboratory. It is described below (Fig. 1).

3. The SRB assay

3.1. Materials, buffers and reagents

Plant extracts and compounds: plant extracts should be from authenticated sources and a voucher specimen kept of every plant collected and extracted. Ideally any extract should be defined so that future extracts made can be compared to check that the composition is the same. TLC or HPLC profiles best define extracts chromatographically, the latter ideally linked to a mass spectrometer, so that LCMS chromatograms can be obtained and significant peaks identified. The identity of isolated compounds should be verified by chromatographic comparison with authenticated reference material or characterised by spectroscopic data, especially those from mass spectra and NMR experiments. Extract and compound solubilisation: Make stock solutions of 40 mg/ml of extract or compounds in an appropriate solvent which will not harm the cells to be used. Dimethyl sulphoxide (DMSO) is often used but, since it is cytotoxic at high concentrations, dilutions with medium must be made so that the final concentration used in treating the cells is below 1% w/v. Tests should be carried out with any solvent used to check its cytotoxicity, using the SRB assay described below. Sterilise all stock solutions by filtration (0.22 μm pore size) and store at −20 °C.

3.1.1. Cell lines

Cell lines used should be defined and obtained from a specified source e.g. European Collection of Cell Cultures (ECACC) or American Type Culture Collection (ATCC). A mixture of cancer cell lines (e.g. COR-L23 human non-small lung cancer, C32 human amelanotic melanoma) and non-cancer cell lines (e.g. MRC-5 human foetal lung fibroblast) should be used.

All cells should be cultured in appropriate medium and supplemented with 10% v/v foetal bovine serum (FBS), 1% w/v penicillin (104 U/ml)/streptomycin (10 mg/ml) and 1% w/v L-glutamine (200 mM). All these reagents were obtained from Sigma–Aldrich, UK.

3.2. Procedures

3.2.1. Harvesting cells

1. Incubate cells in appropriate culture medium at 37 °C in 5% v/v CO_2 until 75% confluent.
2. Rinse cells with 5 ml Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS) and aspirate.
3. Incubate cells with 2 ml 0.05% w/v trypsin/0.5 mM EDTA (Sigma–Aldrich, UK) for 3 min at 37 °C.
4. Tap cell culture flask on the edge with horizontal force until the cells detach. If necessary, incubate the flask for an additional 1 min to allow the cells to detach completely.
5. Triturate the detached cells with a 1 ml pipette several times to disrupt cell clumps and produce a single cell suspension.
6. Add 5 ml medium containing 10% v/v foetal bovine serum to inactivate the trypsin.
7. Transfer the cell suspension to a 15 ml conical tube and centrifuge at 100 g for 5 min.
8. Discard the supernatant and resuspend the cells in culture medium or PBS as appropriate.

3.2.2. Preparation of cells for the assay

Several factors need to be considered before an assay is executed. These include the seeding density, i.e. the correct concentration of cells to be used, and the time over which the assay should be carried out.

The seeding density is dependent on the cell cycle time of each cell line. There are two criteria to be considered for the selection of preferred cell density. First, all cell lines in control must be keep in the exponential growth phase over the incubation time of bioassays. On the other hand, the doubling time (total cell cycle time) must be shorter or equal to the incubation time for some bioassays, otherwise a cell cycle-specific effect may not be observed.

3.2.3. Determination of total cell cycle time

1. Using a haemocytometer to measure the cell density of the original suspension, prepare suspensions of cells in the appropriate growth medium at different densities to give volumes of about 100 μL to contain from 1×10^3 to 7×10^3 cells/well.
2. Seed 100 μL aliquots of cell suspensions into 96-well plates.
3. Incubate for 5 days (120 h) and, using a haemocytometer, measure the cell density of each suspension every 24 h.
4. Plot the log of the density of cells against time to construct a growth curve. A straight line on such a graph will show the exponential proliferation of the cells.
5. The cell cycle time can be calculated from the graph by the slope of the line. A higher seeding density results in a shorter doubling time.
6. A seeding density should be chosen which gives a cell cycle time of about 48 h. At higher densities, contact inhibition of cells occurs and nutrients are exhausted more quickly from the medium.

3.2.4. The SRB assay

The term cytotoxicity covers both cytostatic or cytocidal effects. The SRB assay can be used to determine which of

these takes place for a particular test substance. Exposure to the test substance may result in a reduction of proliferation of cells at the end of the specified time compared to the control wells where no cytotoxic substance has been added. The IC_{50} value determined under these conditions is known as the 'Exposure' dose. At high concentrations no cells remain at this time i.e. all cells have been killed, but if some viable cells remain, it is not possible to know if they are capable of revival and proliferation once the toxic substance is removed.

In order for this to be investigated, the cell medium, which contains the cytotoxic agent, is removed and replaced with fresh medium containing no cytotoxic substance. The cells are then re-incubated for at least the same time as that used for 'Exposure' and the SRB assay carried out. IC_{50} determinations from this second assay are known as 'Recovery' values. If exposure to the extract does not kill the cells, they will revive when the fresh medium is added and any IC_{50} value will be much higher than for the exposure value. If the extract permanently affects the cells then the IC_{50} value will be similar to that shown by the exposure assay.

Materials. Cell lines are cultured in an appropriate medium e.g. for COR L23 cells RPMI 1640 (European Collection of CellCultures, UK) supplemented with 10% v/v foetal bovine serum, 2 mM glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin (all from Sigma–Aldrich, UK). Cell lines are maintained at 37 °C in a 5% v/v CO_2 atmosphere with 95% v/v humidity. Cultures are passaged weekly and the culture medium changed once every 5 days.

Reagents. 40 w/v w/w ice-cold trichloroacetic acid (TCA) (Sigma–Aldrich, UK); 0.4% w/v sulphorhodamine B (SRB) (Sigma–Aldrich, UK) in 1% v/v acetic acid; 1% v/v acetic acid for washing cells; 10 mM Tris[hydroxymethyl]aminomethane buffer (TRIS base).

3.2.4.1. Procedure for SRB exposure assay.

1. Introduce 100 μl of cell suspension of optimum density into each well of a 96-well plates.
2. Make up a range of concentrations of the substances to be tested in the culture medium so that, when diluted 2×, the range of final concentrations in the wells is 100 to 0.2 μg/ml for extracts and 50 to 0.1 μg/ml for compounds. Make up a range of concentrations for a known cytotoxic agent e.g. vincristine sulphate (Sigma–Aldrich, UK), to be used as a positive control.
3. Add 100 μl of each concentration of test sample in culture medium to the wells containing the cells. Add 100 μl medium only to the control wells.
4. Incubate the cells with the samples for 48 h.
5. Fix the cells with ice-cold TCA for 1 h at 4 °C.
6. Wash the plates five times in distilled water and allowed to dry in the air.
7. Add 50 μl sulphorhodamine (SRB) solution to each well of the dry 96-well plates and allow staining at room temperature for 30 min.
8. Remove the sulphorhodamine (SRB) solution by washing the plates quickly with 1% v/v acetic acid, five times, to remove unbound dye.
9. Dry the washed plates in the air. Solubilise the bound SRB by adding 100 μl of 10 mM unbuffered Tris Base (pH 10.5) to each well and shaking for 5 min on a shaker platform.
10. Read the plates in a 96-well plate reader e.g. SpectraMax-190 (Molecular Devices, Sunnydale, USA) with the working wavelength 492 nm.
11. The optical density (OD) of SRB in each well is directly proportional to the cell number so the OD values can be plotted against concentration and the IC_{50} determined by using a program such as GraphPad PRISM (GraphPad, UK).

3.2.4.2. SRB recovery assay

1. Perform steps 1–4 as for the exposure assay above.
2. Wash the plates five times in distilled water and allowed to dry in the air.
3. Introduce 200 μl medium into each well.
4. Incubate for 72 h.
5. Perform the SRB assay as described in steps 5–11 above.

A large number of reports exist of using the SRB assay to screen extracts for cytotoxicity and for bioassay-guided isolation of active compounds e.g. [8–11].

4. Metabolites as active cytotoxic agents

Most cytotoxicity screens have used plant extracts without taking into account the fact that the naturally occurring compounds may not in themselves be active, but may require transformation to active substances by metabolic systems in the body as an explanation of a traditional use. This has been shown to be occur for several traditional medicinal plants, e.g. the laxative drug senna, where the glycosides are hydrolysed by intestinal flora to release the aglycones which are the compounds causing alterations in permeability to water of the gut wall and increase in peristalsis.

Metabolism may also occur in the liver after absorption of compounds from the gut and this is known to occur with the synthetic anticancer drug cyclophosphamide, which is converted to the cytotoxic DNA-alkylating phosphoramide mustard by cytochrome P450 enzymes [12].

It is therefore not unreasonable to suppose that metabolic systems such as these may produce cytotoxic compounds from non-cytotoxics present in the plant. Model systems have been developed in our laboratory to investigate whether this may occur.

4.1. Gut bacterial hydrolysis

Metabolism of ingested materials in the gut is complex due to the range of different bacteria found there and also

the enzymes produced by the intestinal wall. Hence any *in vitro* model must be very reductionist and it must be borne in mind that different individuals will have different gut flora, dependent on diet and geographical location, as well as endemic enzyme profiles dependent on genetics.

The model that has been developed utilises β-glucosidase (Sigma–Aldrich, UK), an enzyme commonly associated with gut bacteria. This enzyme cleaves most glycosides containing an *O*-β-glucose link and it is known that, for several types of molecule, the aglycone thus obtained is more active and bioavailable than the parent glycoside. This has been demonstrated for the flavonoid rutin and its aglycone quercetin [13] and the isoflavone glycosides tectoridin and 6″-*O*-xylosyltectoridin and their aglycone tectorigenin [14]. These reports suggest that some glycosides, especially flavonoid glucosides, should be considered as natural prodrugs in traditional medicine, and that they can be transformed into active compounds by human intestinal bacteria.

A 'one pot' reaction mixture of bacterial culture with extract in a cytotoxicity assay, such as SRB, is difficult because contamination with bacteria can affect the cell growth, and metabolism requires a fairly long time of pre-incubation.

A cytotoxicity assay with a prior enzymatic hydrolysis procedure was therefore established in our laboratory using β-glucosidase, an enzyme found not only in bacterial gut flora but also in human organs [15], to investigate the possibility of β-glucosides acting as prodrugs. The technique is based on the proposition that hydrolysis of β-glucoside flavonoids would happen *in vitro* in a similar way as *in vivo* and is an adaptation of a previously reported procedure for inhibition of cholesterol synthesis pre and post-treatment with enzyme [16].

A check was made that the β-glucosidase was non-toxic to the cultured cells by carrying out the SRB assay for a range of concentrations of the enzyme.

4.1.1. Materials and enzymes

Compounds or plant extracts dissolved in cell culture medium to give a concentration of 100 or 200 μg/ml and sterilised by filtration through a 0.22 μM filter. β-Glucosidase (Sigma–Aldrich Fluka 49290, UK) ⩾6 U/mg in cell culture medium to obtain a 1 mg/ml solution.

The cell culture medium should be appropriate for the cells being used.

4.1.2. Procedure

1. Establish culture of cells in confluence using the appropriate medium.
2. Mix the plant extract with the enzyme solution and incubate at 37 °C for 1 h. Use this solution at different concentrations for the SRB assay.
3. Carry out the SRB assay for the test substances with and without enzyme treatment, using the β-glucosidase solution alone and medium alone as negative controls. Prepare duplicate ranges of concentrations for the test substance. Test one set for cytotoxicity using the SRB assay after the initial 48-h exposure time.
4. For the second set, after the initial 48-h exposure, replace medium with fresh medium and carry out the SRB determination after a further 72-h exposure.

This test was carried out for water and methanol extracts of four Chinese plants used traditionally to treat cancer, as well as on luteolin-7-*O*-glucoside **1** (Extrasynthese, France). The plants were the fruits of *Illicium verum* Hook f. (Illiciaceae), the flowers of *Lonicera japonica* Thunb. (Caprifoliaceae), the seeds of *Dolichos lablab* L. (Fabaceae) and the rhizomes of *Iris tectorum* Maxim. (Iridaceae).

Preliminary tests had shown that the large cell lung carcinoma cell line COR-L23 ECACC no: 92031919 was susceptible to all these extracts. Cells were cultured in RPMI 1640 medium supplemented with 10% v/v heated foetal bovine serum, 1% w/v 200 mM L-glutamine and 1% v/v of 10,000 U/ml penicillin and 10 mg/ml streptomycin. Three replicates were run for each determination. Determinations were carried out for 48 hours' exposure.

The percentage survival of cells was calculated from the optical density readings and results are shown in Table 1.

4.1.3. Results and discussion

The validity of the test is shown by the results obtained for luteolin-7-*O*-glucoside **1**. The untreated compound showed no significant cytotoxicity, but after it was incubated with

Table 1
Cytotoxicity (percentage survival) for Chinese anticancer plants and luteolin-7-*O*-glucoside without and without treatment with β-glucosidase ($n = 3$)

Plant extract (μg/ml)	Exposure 48 h	
	No enzyme	Enzyme
Dolichos lablab water (100)	99.2 ± 1.2	98.5 ± 1.2
Dolichos lablab water (200)	99.0 ± 0.2	99.1 ± 0.3
Dolichos lablab methanol (100)	102.2 ± 6.6	106.4 ± 6.5
Dolichos lablab methanol (200)	92.6 ± 1.1	97.3 ± 0.6
Illicium verum water (100)	97.8 ± 0.2	93.6 ± 0.4
Illicium verum water (200)	95.4 ± 0.3	91.8 ± 0.1
Illicium verum methanol (100)	95.8 ± 0.0	96.0 ± 0.0
Illicium verum methanol (200)	88.5 ± 0.6	95.9 ± 0.3
Iris tectorum water (100)	92.1 ± 4.1	94.2 ± 6.5
Iris tectorum water (100)	97.8 ± 6.3	93.7 ± 8.1
Iris tectorum methanol (100)	78.8 ± 2.1	72.6 ± 1.9
Iris tectorum methanol(200)	63.4 ± 1.5	52.6 ± 0.2*
Lonicera japonica water (100)	79.1 ± 2.1	82.8 ± 6.6
Lonicera japonica water (200)	18.0 ± 2.2	39.2 ± 2.3
Lonicera japonica methanol (100)	64.3 ± 0.1†	86.1 ± 5.3
Lonicera japonica methanol (200)	9.5 ± 0.3†	56.1 ± 1.6
Luteolin-7-*O*-glucoside 15 μM	102 ± 3	53 ± 1*
Luteolin-7-*O*-glucoside 100 μM	96 ± 2	18 ± 1*
Luteolin 15 μM	53 ± 1	60 ± 2
Luteolin 100 μM	19 ± 0.5	21 ± 1

* $P < 0.05$ difference between treated and untreated compound.
† $P < 0.05$ difference between untreated and treated compound.

β-glucosidase, dose-related cytotoxicity was detected which was very similar to that given by the aglycone luteolin **2**.

Neither of the extracts of *I. verum* or *D. lablab* (DL) exhibited cytotoxicity against COR-L23 cells after 48 h exposure, with or without treatment with the enzyme. The methanol extracts of *L. japonica* actually showed less cytotoxicity after enzyme treatment. This implies that compounds that are naturally present are converted into more toxic compounds and raises safety issues about the extracts.

Iris tectorum water extract showed no cytotoxicity before or after β-glucosidase treatment but the methanol extract showed some inherent cytotoxicity, which appeared to be enhanced after enzyme treatment, especially for the 200 μg/ml dose. This indicates that weakly cytotoxic compounds are hydrolysed to more toxic ones. This is not surprising, since tectorigenin glycosides have been shown to be present, and the cytotoxicity of their aglycone tectorigenin **3** has been demonstrated [14].

4.2. Cytochrome P450 metabolism

Cytochrome P450 is found in highest amounts in the liver and is actually a family of different enzymes that metabolise xenobiotics in a range of different ways [17].

The test is a comparison between the cytotoxic activity, assessed by a method such as the SRB assay, of a plant extract or compound before and after incubation with cytochrome P450, usually employed as a suspension of rat liver microsomes. If the IC_{50} value after incubation is much lower than that before, it can be assumed that active compounds have been produced during the incubation process, as long as a control is used which contains the same materials but where enzyme activity has been stopped.

4.2.1. Materials, buffers and enzymes

Dissolve compounds or plant extracts in sterile water to give a concentration of 8 mM or 20 mg/ml, respectively. Mix 40.5 ml dibasic sodium phosphate Na_2HPO_4 200 mM and 9.5 ml monobasic sodium phosphate NaH_2PO_4 200 mM to achieve sodium phosphate buffer, pH 7.4. Cofactor A solution: dissolve magnesium chloride, NADP+ and glucose-6-phosphate (all from Sigma–Aldrich, UK) in the sodium phosphate buffer solution to give concentrations of 200, 10 and 100 mM, respectively. Cofactor B (Sigma–Aldrich, UK) 8 U/ml, glucose-6-phosphate dehydrogenase in sodium buffer. Microsomal suspension prepared by suspending male Wistar rat liver microsomes in 20% v/v glycerol to obtain a final protein concentration of 2 mg/ml. Ten percentage of w/v trichloroacetic acid; 10 M sodium hydroxide solution.

4.2.2. Procedure

1. Mix 1.0 ml of sodium phosphate buffer, pH 7.4, 1.0 ml of test substance solution, 0.5 ml of cofactor A solution and 0.5 ml of cofactor B solution and pre-incubate at 37 °C for 5 min prior to the initiation of the reaction.
2. Add 1.0 ml of the microsomal suspension to the mixture and incubate at 37 °C for a further 30 min.
3. Add 0.5 ml of 10% w/v trichloroacetic acid and then centrifuge at 13,000 rpm for 10 min.
4. Remove supernatant and adjust to pH 7.0 with 10 M sodium hydroxide solution.
5. Use this mixture in a range of dilutions and carry out the SRB assay.
6. Repeat steps 1–5 above but add the 10% w/v trichloroacetic acid after adding the microsomal suspension. This is the control test for the extract.

This test was carried out for eight aqueous extracts of plants used in a traditional Thai remedy for cancer. The plants and their ratio in the preparation are shown in Table 1. Preliminary tests against several different cancer cell lines had shown that the large cell lung carcinoma cell line COR-L23 ECACC No.: 92031919 was the most susceptible. Cells were cultured in RPMI 1640 medium supplemented with 10% v/v heated foetal bovine serum, 1% w/v 200 mM L-glutamine and 1% v/v of 10,000 U/ml penicillin and 10 mg/ml streptomycin (all from Sigma–Aldrich, UK). Cyclophosphamide (Sigma–Aldrich, UK) was used as a positive control. Three replicates were run for each determination. Determinations were carried out for 48 hours' exposure and for 72 h with fresh medium added after the initial 48 hours' exposure.

The IC_{50} values were calculated from the Graphpad Prism programme by plotting a dose response curve between percentages of cell survival (deduced from optical density of the well) against extract concentration.

4.2.3. Results

Results are shown in Table 2. It can be seen that the test is validated because the cyclophosphamide is significantly more active ($P < 0.05$) after incubation with the microsomal cytochrome P450 preparation. However, none of the extracts tested displayed a significant difference between treated and untreated extract, either with initial exposure or with the recovery test, thus indicating that any active substances e,.g. in the *Ammania baccifera* extract, which gives the lowest IC_{50} value, are present naturally, and that no more active compounds are formed in any of the extracts after incubation (Table 3).

5. Test for activities related to prevention of carcinogenesis

Over the last two decades, there has been much interest in the role of diet and natural substances in prevention of cancer. Much attention has been focussed on ways in which the antioxidant activity of compounds might prevent cancer by inhibiting damage to DNA and cell membranes and several tests have been described to detect inherent antioxidant effects in extracts and compounds. Such tests are not covered in this paper since a plethora of relevant reviews and reports exists e.g. [18–22].

Table 2
Plants used in traditional Thai treatment for cancer

Plant	Family	Part of plant used	Percentage weight in mixture
Canna indica L.	Cannaceae	Rhizome	52.7
Ammania baccifera L.	Lythraceae	Whole plant	7.9
Acanthus ebracteatus Vahl.	Acanthaceae	Whole plant	7.9
Clinacanthus nutans Lindau	Acanthaceae	Leaf	7.9
Mallotus phillippinensis Muell. Arg.	Euphorbiaceae	Stem	7.9
Polygala chinensis L.	Polygalaceae	Whole plant	7.9
Premna herbacea Roxb. Mold.	Verbenaceae	Rhizome	3.9
Smilax corbularia Kunth.	Smilacaceae	Rhizome	3.9

Table 3
Cytotoxicity (IC_{50} values μg/ml) for Thai plant extracts and cyclophosphamide against COR-L23 cancer cell line with and without microsomal incubation ($n = 3$)

Plant	Exposure 48 h		Recovery 72 h with fresh medium after initial 48 h exposure	
	Incubation	No incubation	Incubation	No incubation
Canna indica *L.*	67.84 ± 5.62	68.65 ± 14.26	36.14 ± 1.54	36.49 ± 0.79
Ammania baccifera L.	56.39 ± 4.56	49.45 ± 1.46	52.73 ± 4.04	46.90 ± 1.70
Acanthus ebracteatus Vahl.	126.04 ± 2.81	126.85 ± 9.96	109.82 ± 0.23	110.35 ± 2.84
Clinacanthus nutans Lindau	171.36 ± 4.48	181.08 ± 2.37	114.11 ± 7.27	124.99 ± 3.29
Mallotus phillippinensis Muell. Arg.	66.82 ± 6.90	71.87 ± 2.02	37.29 ± 1.25	35.17 ± 0.92
Polygala chinensis L.	99.07 ± 9.58	98.78 ± 6.99	43.23 ± 2.91	44.23 ± 3.35
Premna herbacea Roxb. Mold.	>200	>200	>200	>200
Smilax corbularia Kunth.	>200	>200	>200	>200
Cyclophosphamide	0.398 ± 0.034*	0.507 ± 0.021	0.099 ± 0.01*	0.467 ± 0.012

* $P < 0.05$.

The problem with many antioxidants proposed as cancer preventive agents is that little is known of their bioavailability i.e. whether they reach the target cells and tissues in sufficient concentration to exert activity. An alternative approach is to consider compounds which might effect the mobilisation of the cell's own defence mechanisms against oxidative and other damage. Less attention has been paid to tests for this type of activity but a method for testing upregulation of glutathione-*S*-transferase (GST) has recently been introduced in our laboratories.

5.1. Upregulation of GST

The glutathione-*S*-transferase (GST) enzymes catalyse the nucleophilic addition of glutathione (GSH) to an electrophilic centre found in numerous xenobiotics. The GST isoenzymes do not normally operate at their maximal capacity, but can be transcriptionally activated (induced) by a wide variety of natural and synthetic chemical agents, thereby achieving efficient protection against carcinogenesis [23]. The GST isoenzymes also induce a variety of other systems such as NAD(P)H:quinone reductase (NQO1), catalase, superoxide dismutase and GST efflux pumps [24]. GSTs deactivate cytotoxic and genotoxic compounds through catalysing the *S*-conjugation of the electrophilic moiety with reduced glutathione (GSH). In the test that has been developed, the levels of GSH concentrations and cell viability in *in vitro* cell cultures are measured. HepG2 was used as an *in vitro* model cell line to investigate this, as it is highly differentiated and has retained many of the specialised functions normally lost by hepatocytes in culture [25].

5.1.1. Materials

Plant species with a reputation of being used as traditional anticancer remedies in Malaysia and Thailand were used together with three phenylpropanoid compounds **4–6** isolated from the *Alpinia* species investigated [11]. Extracts used have been reported previously and test samples were diluted as required in culture medium to reach a final solvent concentration of not more than 0.5% (v/v), the test samples at concentrations of 2.5, 25.0 and 250 μg/ml (for plant extracts) and a range of 0.625 to 25 μg/ml (for pure compounds).

Phosphate-buffered saline (PBS) pH 7.4; 1 M potassium dihydrogen phosphate solution containing EDTA; OPT reagent: 1% w/v *o*-phthaldialdehyde freshly prepared in methanol and stored in the dark (all from Sigma–Aldrich, UK). GSH solution: pure glutathione (GSH) (Sigma–Aldrich, UK) dissolved in 1% v/v perchloric acid to give a range of concentrations 2.5 to 20.0 nmol/ml. CDNB solution: 30 mM 1-chloro-2,4-dintrobenzene in PBS. Two millimolar phenobarbitone sodium in PBS (all from Sigma–Aldrich, UK).

Cell culture. HepG2 cells (supplied by GSK, UK) were grown in MEM (Eagle's Minimum Essential Medium), supplemented with 10% v/v foetal bovine serum, 1% v/v non-essential amino acid, 2 mM glutamine, 50 IU/ml

penicillin and 50 μg/ml streptomycin (all from Sigma–Aldrich, UK). Cells were maintained at 37 °C in a 5% CO_2 atmosphere with 95% humidity. Maintenance cultures were passaged every 5 days using (10×) trypsin–EDTA (Sigma–Aldrich, UK), and the culture medium was changed every 3 days. Cells were regularly monitored for the presence of mycoplasma infection with the *Mycoplasma Testing Kit* (Roche Diagnostics, UK). Cells were detached with (10×) trypsin/EDTA (Sigma–Aldrich, UK) to make single-cell suspensions. Viable cells were counted by trypan blue exclusion in a haemocytometer and diluted in medium to give a final concentration of 1.0×10^5 cells/ml.

5.1.2. Procedure for preparation of cytosolic solution

1. Seed 1.0 ml of cell suspensions in 24-well plates and incubate to allow for cell attachment for 24 h.
2. Aspirate cell media from the 24-well plates. Wash cells attached to the surface twice with 1.0 ml phosphate-buffered saline (PBS).
3. Add 1.0 ml of test solution the wells in duplicate. Add 1.0 ml of solvent to the control wells.
4. Leave treated cells for 48 h.
5. Aspirate test solutions from wells, wash cells twice with PBS then detach from wells using 0.1 ml (10×) trypsin/EDTA.
6. Add 0.9 ml of fresh media to inhibit trypsin activity.
7. Transfer cells into 1.5 ml microcentrifuge tubes and centrifuge at 10,000*g* for 5 min.
8. Remove media, rinse once with 0.5 ml PBS and re-centrifuge.
9. Remove PBS and then suspend cell pellet in 0.5 ml 0.1 M potassium phosphate buffer at pH 6.5.
10. Lyse by sonication for 10 s and then maintain cell suspension at 4 °C.
11. Add trypan blue and examine cells microscopically to check that cells are lysed.
12. Centrifuge lysed suspension at 10,000*g* for 5 min.
13. Use the cytosolic supernatant to measure GST activity and GSH concentration.

5.1.3. Assay of GST activity

1. Pre-incubate the cytosolic cell fraction for 5 min at 37 °C.
2. Add 8.3 mM GSH and 3.3 mM CDNB and record the increase in absorbance of the solution at 30 °C for a further 10 min using a plate reader.
3. Assay for protein concentration using the method of Bradford [26], using bovine serum albumin as a standard. Carry out at least three independent experiments in duplicate wells for each test sample. Use potassium phosphate buffer instead of cytosolic cell fractions in the control wells.
4. Express the specific GST activity in units per mg cell protein using the equation.

$$\text{Specific activity} = \frac{\text{absorbance rate per min} \times \text{measurement time(min)}}{9.6\,\text{mM}^{-1}\,\text{cm}^{-1} \times \text{protein concentration(mg/ml)}}$$

One unit of activity is defined as the amount of enzyme catalysing the conjugation of 1 μmol CDNB/min/mg at 30 °C.

Two millimolar phenobarbitone sodium, a known inducer of all the GST subunits and CYP450 enzymes is used as a positive control.

5.1.4. Assay for GSH levels

1. Grow HepG2 cells under identical conditions to those used for the assessment of GST activity.
2. Inoculate cells onto 24-well plates and treat with test samples in a similar manner and concentrations to those described above, using three 24-well plates for each compound.
3. Incubate one plate for 8 h, one plate for 24 h and a third plate for 48 h.
4. At the end of each incubation period, take out each 24-well plates and remove test solution from wells.
5. Wash cells twice with 1 ml ice-cold PBS and "air-dry".
6. Add 500 μl of 1% v/v perchloric acid (PCA) to each well and leave on ice for 10 min. Cell proteins are precipitated to the base of the wells and GSH in the cell cytoplasm is extracted into the PCA.
7. Transfer PCA extract to microcentrifuge tubes and ultracentrifuge at 10,000*g* at 4 °C for 5 min.
8. Add 7.5 μl of PCA extract to duplicate 96-wells containing 275 μl of phosphate 0.1 M KH_2PO_4/EDTA buffer.
9. Add 15 μl of 1% w/v OPT reagent (freshly prepared in methanol and stored in the dark) to each well.
10. Leave to react for 25 min at room temperature.
11. Measure for fluorescence (activation peak at 350 nm and fluorescence emission peak at 420 nm), using a Multi-well Plate Reader (Series 4000 CytoFluor®, USA).
12. Obtain a standard curve for each of the plates used as described in steps 13–15.
13. Dissolve pure GSH in 1% v/v PCA to produce a stock solution of 30 nmol/ml GSH.
14. Dilute the stock solution to produce six concentrations from 20.0 to 2.5 nmol/ml.
15. Draw the standard curve for fluorescence as a function of GSH concentration using the conditions above in step 11.
16. Solubilise cell proteins precipitated by the PCA in the 24-wells in 0.5 ml (1 M) NaOH and determine total protein concentration of the cells in each well using an established method [26].
17. Express results from each sample as a ratio of mean of GSH levels in treated samples over mean of control samples. Therefore, the value of control is 1.

5.1.5. Cell viability assay

Cell survival was assessed using the uptake of Neutral Red by lysosomes and Golgi bodies to quantitate cell numbers [27].

1. Inoculate HepG2 cells onto 24-well plates and treat with test samples as described above for 48 h.
2. Dilute compound in growth medium to produce final concentrations ranging from 0.625 to 25 μg/ml. The final concentration of the solvent (DMSO) was 0.2% v/v.
3. Treat control wells with 0.2% v/v DMSO in growth medium only.
4. At the end of 48 h of treatment, remove test solutions from the wells by aspiration.
5. Wash cells twice with 1 ml PBS and then treat them with 1 ml of sterile-filtered Neutral Red (Sigma–Aldrich, UK) solution 0.01% w/v dissolved in growth medium.
6. Incubate cells at 37 °C for 2 h to allow viable cells remaining in the wells to absorb the Neutral Red dye.
7. Discard remaining solution from the wells. Wash remaining cells twice with PBS and dry.
8. Add 1 ml of 50% v/v EtOH in 1% v/v HOAc to each well.
9. Shake plates containing wells gently in a gyratory shaker for 10 min.
10. Transfer each sample in the wells into duplicate wells in a 96-serowell plate to read absorbance at 540 nm using a plate reader.
11. Calculate percentage cell survival as a percentage absorbance of treated wells compared with the mean absorbance of control wells.
12. Obtain the IC_{50} value of compounds by interpolation at the 50% of a dose–response cubic spline curve using GraphPad Prism 2.0.

5.2. Results and discussion

Theza GST activity of HepG2 cells was expressed as the percentage of activity of cells exposed to test sample compared to the activity shown by the negative control. Significant induction of activity in comparison with control was given some of the extracts (Table 4). The most active extracts were (in decreasing order of activity) the CH_2Cl_2 extracts of *A. officinarum* (2.5 μg/ml inducing 138%), (Thai) *Alpinia galanga* (25.0 μg/ml inducing 1.65%), *Jasminum sambac* (25.0 μg/ml inducing 1.61%), (Malaysian) *A. galanga* (25.0 μg/ml inducing 138%) and *Cayratia japonica* (25.0 μg/ml inducing 134%). Phenobarbitone sodium (2nM), the positive control, induced activity by 156%. The results of the crude extract of *A. galanga* are in agreement with previous *in vivo* studies of galanga root oil [28,29]. 1′-Acetoxychavicol acetate **4** caused the strongest induction of GSH levels at early exposure (Table 5), which was followed by a reduction at longer exposures and was the most active GST inducer out of the three compounds from *A. officinarum*. *Trans-p*-coumaryl diacetate **5** exhibited a similar profile of induction effects, but at a lower level

Table 4
GST induction activity on Hep G2 cells of Malaysian and Thai plant extracts

Plant extract	Mean activity ratio ± SEM of test sample (μg/ml)		
	2.5	25.0	250.0
Thai *Alpinia officinarum* in MeOH	1.21 ± 0.07	NA	NA
Thai *Alpinia officinarum* in CH_2Cl_2	1.38 ± 0.11*	1.86 ± 0.14**	NA
Thai *Alpinia officinarum* in H_2O	1.08 ± 0.09	1.03 ± 0.10	1.04 ± 0.08
M'sian *Alpinia galanga* in MeOH	1.16 ± 0.04	1.29 ± 0.14	NA
M'sian *Alpinia galanga* in CH_2Cl_2	1.09 ± 0.09	1.38 ± 0.10*	NA
M'sian *Alpinia galanga* in H_2O	1.43 ± 0.27	1.08 ± 0.11	1.29 ± 0.03
Thai *Alpinia galanga* in MeOH	1.22 ± 0.10	1.23 ± 0.10	1.55 ± 0.16**
Thai *Alpinia galanga* in CH_2Cl_2	1.14 ± 0.06	1.65 ± 0.17**	1.55 ± 0.15**
Thai *Alpinia galanga* in H_2O	1.36 ± 0.11	1.27 ± 0.11	1.40 ± 0.22
Cayratia japonica in MeOH	1.28 ± 0.19	1.10 ± 0.22	1.19 ± 0.16
Cayratia japonica in CH_2Cl_2	1.08 ± 0.11	1.34 ± 0.14*	1.90 ± 0.07**
Cayratia japonica in H_2O	1.02 ± 0.29	1.03 ± 0.22	1.00 ± 0.11
Jasminum sambac in MeOH	1.34 ± 0.09	1.18 ± 0.11	2.24 ± 0.62*
Jasminum sambac in CH_2Cl_2	1.35 ± 0.16	1.61 ± 0.21*	NA
Jasminum sambac in H_2O	1.05 ± 0.22	1.04 ± 0.11	1.55 ± 0.22
Physalis minima in MeOH	0.87 ± 0.09	1.04 ± 0.09	0.90 ± 0.06
Physalis minima in CH_2Cl_2	1.44 ± 0.15	0.95 ± 0.09	1.17 ± 0.09
Physalis minima in H_2O	1.01 ± 1.34	1.03 ± 0.24	1.29 ± 0.16
Tabernaemontana divaricata in MeOH	1.03 ± 0.05	1.15 ± 0.05	NA
Tabernaemontana divaricata in CH_2Cl_2	1.02 ± 0.13	1.14 ± 0.06	NA
Tabernaemontana divaricata in H_2O	0.82 ± 0.40	1.13 ± 0.06	0.93 ± 0.09
Phenobarbitone sodium positive control	1.56 ± 0.05** (2 mM)		

Induction results expressed as a mean of ratio of GST activity of treated vs. solvent control samples. Significant values are denoted by * for $P < 0.05$ and ** for $P < 0.0$, in comparison to the control. The activity ratio of control is 1.
$n = 6$; NA, no results obtained.

Table 5
GSH expressed as percentage of treated cells over solvent control cells, from different periods of exposure to pure compounds

Compound	Mean ratio of GSH levels (%) ± SEM (μg/ml)			
	0.625	6.25	12.5	25
4				
8 h	143 ± 15	169 ± 36**	172 ± .25**	118 ± 17
24 h	99 ± 8	83 ± 7	91 ± 11	102 ± 15
48 h	107 ± 17	94 ± 12	64 ± 7*	80 ± 12*
5				
8 h	89 ± 5	96 ± 8	97 ± 12	78 ± 7*
24 h	108 ± 7	128 ± 14	136 ± 25**	139 ± 31**
48 h	95 ± 4	110 ± 7	92 ± 6	82 ± 6
6				
8 h	87 ± 5	82 ± 7	88 ± 8	80 ± 5*
24 h	109 ± 10	140 ± 7**	152 ± 5**	166 ± 19**
48 h	93 ± 4	98 ± 3	127 ± 11	110 ± 8

Significant values are denoted by *(for $P < 0.05$) and **(for $P < 0.01$), in comparison to the control cells, $n = 4$.

and longer exposure. Structure–activity comparison of the two compounds indicated that 1′-acetoxychavicol acetate **4** possesses a more electrophilic carbon centre than *trans-p*-coumaryl diacetate **5**. This factor could explain the difference in activities between the two compounds.

The phenylpropanoid constituents of *A. officinarum*, 1′-acetoxychavicol acetate **4** and *trans-p*-coumaryl diacetate**5** displayed some inductive activity but another similar compound 4-hydroxycinnamaldehyde **6**, did not (Table 5). This indicates that **4** and **5** are major compounds responsible for the activity demonstrated by the *A. officinarum* extract.

The GSH levels in the cells after exposure to the compounds from the *A. officinarum* are shown in Table 5. It can be seen that the time taken for effects to be observed is much shorter than for the GST activity. *Trans-p*-coumaryl diacetate **5** demonstrates weaker overall effects than 1′-acetoxychavicol acetate **4** but **4** exhibited a higher (**A**)/(**B**) ratio (2.09) than **5**. It may be postulated that the induction of GST activity at 3.125 μg/ml may lead to the utilisation of GSH, causing levels to return to normal at 48 hours' exposure.

1′-Acetoxychavicol acetate **5** exhibited pronounced induction of GSH levels at 8 h but the levels decreased following longer exposure. Such significant induction at such short period (8 h) indicates the possibility of direct interaction on the cells. Cellular stores of GSH can be depleted through the glutathione transferase reaction, in which a glutathione molecule is conjugated to the test compound. At the treatment time of 8 h GST activity would not yet be affected, as this happens via numerous and complex pathways involving the transcription of genes. At 48 h exposure, the GST activity was induced to 135% of control, but cell survival was reduced to 37.8%. The inverse dose-dependent relationship between GSH and GST at 48 h might be explained by the fact that the depletion of GSH was the signal to induce GST.

4-Hydroxycinnamaldehyde **6** significantly induced GSH levels to 140% of control at 24 h exposure (6.25 μg/ml). GST activity and cell survival at 48 h were not significantly altered at the concentrations tested. The induction of GSH levels as an effective defence mechanism against the effects of **6** seemed to be well illustrated in this case.

It is of interest that compounds **4** and **5** have also been shown to be cytotoxic in the SRB assay [11] and may thus play a dual role in the treatment of cancer with *Alpinia* species which contain them.

6. Concluding remarks

A very large number of plant extracts have been screened for cytotoxic effects against cancer cell lines over the last twenty-five years and have resulted in some significant drugs being introduced, paclitaxel probably taking pride of place. In addition, the traditional use of a considerable number of plants for cancer has been justified to some extent by the findings that have shown that their extracts are cytotoxic, especially if selectivity is demonstrated, either between different cancer cell lines or between cancer and non-cancer cell lines.

These successes have resulted from testing of some extracts or derived compounds, with little regard to the possible metabolism of constituents *in vivo* or to activities other than cytotoxicity that might reduce carcnogenesis. The tests described above offer some suggestions for the re-investigation of extracts which do not display much direct cytotoxic activity and which have been abandoned or neglected in previous work, as well as indicating novel approaches to complement standard cytotoxic screening procedures for research on previously uninvestigated material.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymeth.2007.01.003.

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