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ORIGINAL ARTICLE

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Inhibition of P-glycoprotein activity and reversal of cancer multidrug resistance by *Momordica charantia* extract

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Abstract *Purpose*: Multidrug resistance (MDR) is known as a problem limiting the success of therapy in patients treated long term with chemotherapeutic drugs. The drug resistance is mainly due to the overexpression of the 170 kDa P-glycoprotein (Pgp), which causes a reduction in drug accumulation in the cancer cells. In this study, novel chemical modulator(s) from bitter melon (*Momordica charantia* L.) extracts obtained from leaves, fruits and tendrils were tested for their abilities to modulate the function of Pgp and the MDR phenotype in the multidrug-resistant human cervical carcinoma KB-V1 cells (high Pgp expression) in comparison with wildtype drug-sensitive KB-3-1 cells (lacking Pgp). *Methods*: The KB-V1 and KB-3-1 cells were exposed to bitter melon extracts in the presence of various concentrations of vinblastine, and cytotoxicity was assessed by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Relative resistance was calculated as the ratio of the IC_{50} value of the KB-V1 cells to the IC_{50} value of the KB-3-1 cells. Accumulation and efflux of vinblastine in KB-V1 and KB-3-1 cells were measured using a [^{3}H]-vinblastine incorporation assay. *Results*: The leaf extracts increased the intracellular accumulation of [^{3}H]-vinblastine in KB-V1 cells in a dose-dependent manner, but extracts from the fruits and tendrils had no effect. By modulating Pgp-mediated vinblastine efflux, the leaf extracts decreased the [^{3}H]-vinblastine efflux in KB-V1 cells in a dose-dependent manner, but not in KB-3-1 cells. Treatment of drug-resistant KB-V1 cells with bitter melon leaf extracts increased their sensitivity to vinblastine, but similar treatment of KB-3-1 cells showed no modulating effect. The fruit and tendril extracts did not affect the MDR phenotype in either cell line. *Conclusion*: The leaf extracts from bitter melon were able to reverse the MDR phenotype, which is consistent with an increase in intracellular accumulation of the drug. The exact nature of the active components of bitter melon leaf extracts remains to be identified.

Keywords KB-V1 cells · P-glycoprotein · Multidrug resistance · *Momordica charantia* · Bitter melon · Biochemical modulator

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Introduction

Resistance of cancer cells to multiple chemotherapeutic drugs, a mechanism termed multidrug resistance (MDR), is a major obstacle to the success of cancer chemotherapy and is closely associated with treatment failure. One major cause of drug resistance is overexpression of the 170 kDa plasma membrane P-glycoprotein (Pgp). Pgp is a member of the highly conserved superfamily of ATP-binding cassette (ABC) transporter proteins. It acts as an ATP-driven efflux pump that decreases intracellular drug accumulation, thereby decreasing the effectiveness of many chemotherapeutic agents [12]. In general, agents used to antagonize MDR alter the drug accumulation defect present in MDR cells, but have little or no effect on drug-sensitive cells. Many pharmacologic agents from diverse structural classes have been identified as Pgp chemosensitizers [6, 10]. However, synthetic MDR-reversing agents are either toxic or not clinically usable [25].

Many efforts have been made to discover new MDR-reversing agents from natural products or plant-derived chemicals. For example, the methanol extract of rosemary (*Rosmarinus officinalis* Labiatae) increases the intracellular accumulation of doxorubicin and vinblastine in drug-resistant MCF-7 human breast cancer cells which express Pgp [19]. Our previous study demonstrated that commercial curcumin (Sigma) inhibits the expression of Pgp in the MDR human cervical carcinoma KB-V1 cells and increased rhodamine-123 (Rh123)

accumulation and inhibits Rh123 efflux in these cells [2]. However, curcumin has a poor bioavailability although it is nontoxic. We therefore investigated other phytochemicals which might inhibit Pgp activity and reverse MDR. Extracts of bitter melon (*Momordica charantia*; family Cucurbitaceae) have been shown to possess antitumor activity [11, 24], antioxidant activities [23], and antiviral (HIV inhibitor) [13], antidiabetic and immunomodulating properties [9]. Therefore, extracts from various parts of bitter melon, a commonly used herb in folk medicine, were investigated for their possible inhibitory effect on Pgp-mediated efflux. In the study reported here, we demonstrate, for the first time, that leaf extracts from bitter melon inhibited Pgp-mediated drug efflux, resulting in an increase in the intracellular accumulation and cytotoxicity of chemotherapeutic drugs in drug-resistant human cervical carcinoma cells in vitro.

Materials and methods

Chemicals

[^{3}H]-Vinblastine (10.8 Ci/mmol) was obtained from Amersham Bioscience (Cardiff, UK). Cyclosporin A, verapamil, vinblastine, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), HRP-conjugated goat anti-mouse IgG and mouse monoclonal anti-Pgp (MDR) clone F4 were obtained from Sigma Chemical Company (St Louis, Mo.). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco BRL (Grand Island, N.Y.). A SuperSignal detection kit was purchased from Pierce (Rockford, Ill.).

Preparation of bitter melon extracts

Whole plant bitter melon plant was harvested from the plantation of Lampang Herb Conservation Assembly in Lampang, Thailand. Natural products, such as bitter melon, are certified by botanists at Lampang Herb Conservation Assembly. Fresh leaves, fruits and tendrils of bitter melon were dried at 30–45°C and ground. Dried powdery plant samples (1 kg) were extracted exhaustively with 4 l of 80% ethanol by maceration at 37°C for 16 h. The mixture was filtered and re-extracted with 4 l of ethanol. The combined filtrate was bleached with 160 g of active charcoal, filtered and concentrated by rotary evaporation to 120 ml before filtering to remove precipitates. The filtrate was rotary evaporated and lyophilized to dryness to produce the bitter melon extract. Bitter melon extract was dissolved in DMSO, and the final DMSO concentration used for experimentation was adjusted to 0.1% (v/v) in the culture medium.

Cells and cell culture

A MDR cervical carcinoma cell line (KB-V1) and a drug-sensitive cervical carcinoma cell line (KB-3-1) were generous gifts from Dr Michael M. Gottesman (National Cancer Institute, Bethesda, Md.). Both cell lines were cultured in DMEM with 4.5 g of glucose/l plus 10% fetal calf serum (FCS), 5 m *M* L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin; 1 μg/ml of vinblastine was added only to the KB-V1 culture medium. These two cell lines were maintained in a humidified incubator with an atmosphere comprising 95% air and 5% CO_2 at 37°C. When the cells reached confluency, they were harvested and plated either for subsequent passages or for drug treatments.

Western blot analysis of Pgp

For Western blotting, plasma membrane from KB cells was prepared according to the method described by Anuchapreeda et al. [2]. The protein amount was measured by the method of Lowry et al. [17] using bovine serum albumin (BSA) as a standard. The plasma membrane proteins (20 μg/lane) were separated on a 7.5% SDS-polyacrylamide gel and immunoblotted overnight onto nitrocellulose filters (GIBCO-BRL). The filters were incubated sequentially with mouse monoclonal anti-Pgp clone F4 [5] at dilution of 1:5000 and HRP-conjugated goat anti-mouse IgG at a dilution of 1:20,000. Proteins were visualized using a SuperSignal protein detection kit and quantitated by scanning densitometry.

Measurement of vinblastine cytotoxicity

KB-V1 and KB-3-1 cells were plated out at 3.0×10^3 cells per well in 96-well plates. After 24 h, medium containing 0.1% DMSO (control), or 25 or 75 μg/ml of bitter melon extract and various concentrations of vinblastine was added. The cells were incubated for 3 days at 37°C, and cell growth was assessed by means of the MTT colorimetric assay [1]. In each experiment, determinations were carried out in triplicate. Relative resistance was calculated as the ratio of the IC_{50} value of the KB-V1 cells to the IC_{50} value of the KB-3-1 cells.

Drug accumulation and efflux assays

Accumulation of vinblastine in KB-V1 and KB-3-1 was measured using a [^{3}H]-vinblastine incorporation assay [19]. KB-V1 or KB-3-1 cells (6.0×10^5 cells/well) were cultured in complete DMEM in six-well plates for 24 h. Cells were treated with various concentrations of bitter melon extracts and the reversing agent (10 μ*M* cyclosporin A) in the presence of 0.05 μCi [^{3}H]-vinblastine/ml for 60 min. After washing with ice-cold PBS (pH 7.4), the cells were harvested, and the amount of intracellular radioactivity was measured.

For determination of drug efflux, cells were plated out as described for the drug accumulation experiments.

Cells were incubated for 60 min at 37°C with 0.05 μCi [^{3}H]-vinblastine/ml in the presence of 50 μ*M* verapamil in order to load cells with radiolabeled drug. Cells were then washed with ice-cold PBS (pH 7.4), followed by medium containing various concentrations of bitter melon extracts and the reversing agent (10 μ*M* cyclosporin A). After incubation at 37°C for 30 min, cells were washed with ice-cold PBS (pH 7.4) and harvested. The amount of intracellular radioactivity was measured.

Statistical analysis

The results are presented as means ± SD from triplicate samples of three independent experiments. Differences between the means were analyzed by one-way analysis of variance. Statistical significance was considered when $P < 0.05$.

Results

Western blot analysis showed that the 170 kDa Pgp was expressed in KB-V1 cells treated with vinblastine at both 0.5 and 1 μg/ml, and the expression level of Pgp correlated well with the elevated concentration of drug. KB-3-1 cells did not express Pgp at a level detectable by the method used in this experiment (Fig. 1). KB-V1 cells overexpressed Pgp, resulting in a 1467-fold increase in vinblastine resistance compared to the wildtype KB-3-1 cells (Table 1).

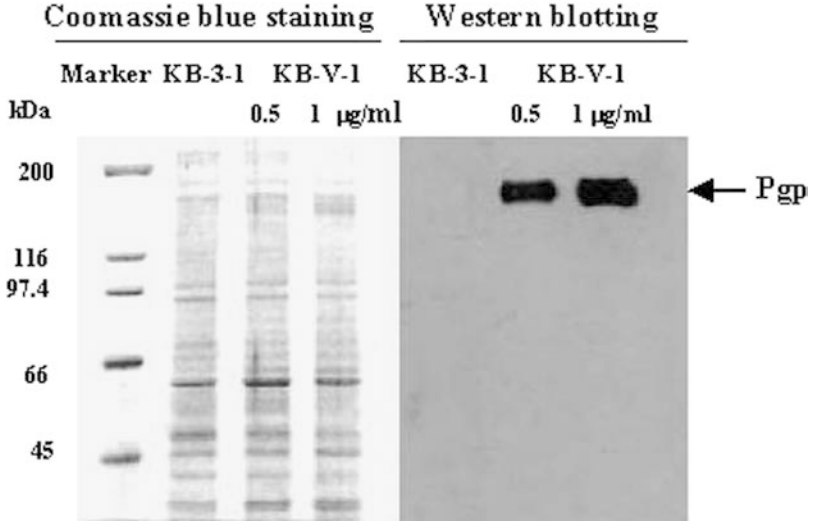
Fig. 1 Pgp expression in the drug-resistant cell line, KB-V1 and drug-sensitive cell line, KB-3-1. KB-3-1 cells were cultured in DMEM without vinblastine. KB-V1 cells were cultured in DMEM with 0.5 or 1 μg/ml vinblastine. Cells were then grown to 80% confluence in T-75 cm^2 culture flasks. Cells were then harvested by scraping and homogenized. The plasma membrane proteins (20 μg/lane) were separated on a 7.5% SDS-PAGE. Pgp was determined by Coomassie blue staining (*left*) and Western blotting using mouse monoclonal anti-Pgp F4 (Sigma-Aldrich) at a dilution of 1:5000 as a primary antibody and HRP-conjugated goat anti-mouse IgG at a dilution of 1:20,000 as a secondary antibody (*right*)

The bitter melon extracts of leaves, fruits and tendrils were studied for their ability to modulate the MDR phenotype in KB cells. Noncytotoxic concentrations (> 80% cell survival) of all plant extracts were used in combination with various concentrations of vinblastine. The modulating effect of bitter melon extracts on vinblastine cytotoxicity was examined by the MTT assay. In KB-V1 drug-resistant cells, coincubation with vinblastine and the leaf extracts resulted in a significant dose-dependent increase in the cytotoxicity of vinblastine (Fig. 2a). Incubation of wildtype KB-3-1 cells with increasing amounts of vinblastine resulted in a decrease in cell growth that was not affected by incubation with the leaf extracts (Fig. 2b). The fruit and tendril extracts had no effect on vinblastine sensitivity in drug-resistant KB-V1 or drug-sensitive KB-3-1 cells lines (data not shown). The IC_{50} values of vinblastine and the relative resistance in KB-V1 cells after treatment with bitter melon extracts are summarized in Table 1. In the presence of 75 μg/ml leaf extract, the IC_{50} of vinblastine to KB-V1 cells was reduced more than threefold. These results demonstrate that bitter melon leaf extract is an effective modulator in restoring the sensitivity of

Table 1 Modulation of resistance to vinblastine in KB cells by bitter melon extracts

	IC_{50}[a]	Relative resistance[b]
KB-3-1	1.5 ± 0.3 n *M*	1
KB-V1	2.2 ± 0.2 μ*M*	1467
KB-V1 + 25 μg/ml leaf extract	1.8 ± 0.1 μ*M*	1200
KB-V1 + 75 μg/ml leaf extract	0.7 ± 0.3 μ*M*	467
KB-V1 + 25 μg/ml fruit extract	2.2 ± 0.1 μ*M*	1467
KB-V1 + 75 μg/ml fruit extract	2.2 ± 0.1 μ*M*	1467
KB-V1 + 25 μg/ml tendril extract	2.1 ± 0.5 μ*M*	1400
KB-V1 + 75 μg/ml tendril extract	2.0 ± 0.3 μ*M*	1333

[a]Determined by the MTT assay, and the values are means ± SD of three independent experiments.
[b]IC_{50} of KB-V1/IC_{50} of KB-3-1.



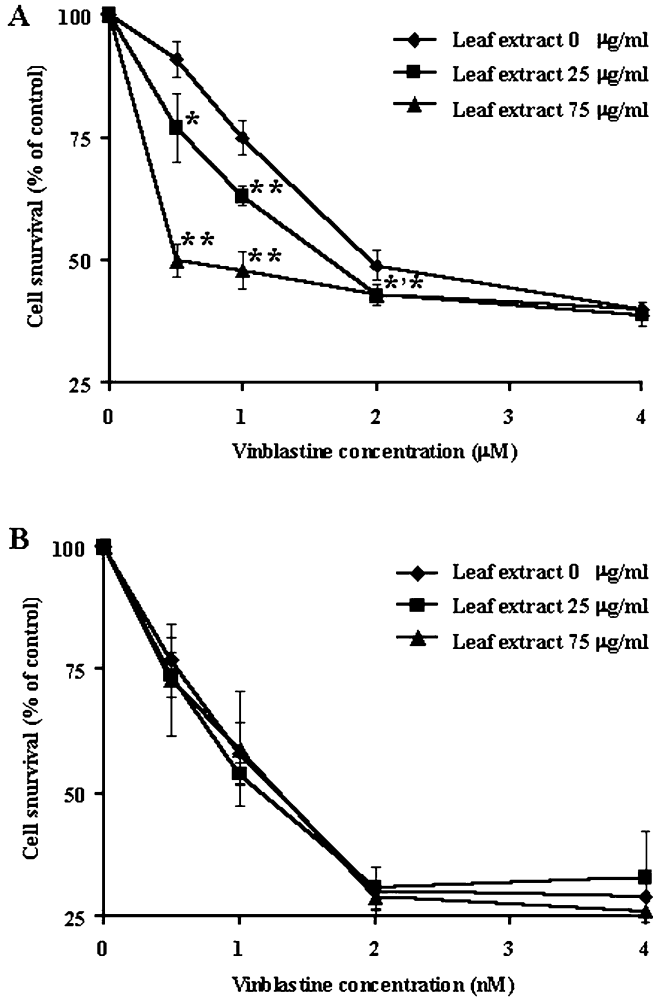


Fig. 2a, b Effect of bitter melon leaf extract on vinblastine cytotoxicity in (**a**) KB-V1 and (**b**) KB-3-1 cell lines. Cells were incubated in the presence and absence of the leaf extract in combination with vinblastine. The number of viable cells was determined by the MTT assay. Each point represents the mean (±SD) of three independent experiments performed in triplicate. $*P < 0.05$, $**P < 0.01$, vs control treated without leaf extract

drug-resistant cells to an anticancer drug in human Pgp MDR cells.

Since the bitter melon leaf extracts enhanced cytotoxicity of vinblastine in multidrug-resistant KB-V1 cells but not in the parental drug-sensitive KB-3-1 cells, we further examined the effects of the leaf extracts on Pgp function. We examined the effect of bitter melon extracts on the intracellular accumulation of radiolabeled drug, [^{3}H]-vinblastine, in drug-resistant KB-V1 cells compared to its accumulation in the drug-sensitive KB-3-1 cells. The leaf extracts increased [^{3}H]-vinblastine accumulation in KB-V1 cells in a dose-dependent manner (Fig. 3a). The presence of leaf extracts at 75 and 100 µg/ml increase [^{3}H]-vinblastine accumulation in KB-V1 cells by about 6- and 16-fold, respectively ($P < 0.05$). In comparison, cyclosporin A at 10 µ*M* enhanced vinblastine accumulation by 11-fold demonstrating that the leaf extracts at 100 µg/ml inhibited drug accumulation more potently than 10 µ*M* cyclosporin A (Fig. 3a). Accumulation of [^{3}H]-vinblastine in drug-sensitive KB-3-1 cells treated with bitter melon leaf extracts or cyclosporin A did not differ from its accumulation in cells without any treatment. There was no change in intracellular [^{3}H]-vinblastine accumulation in KB-V1 cells in the presence of fruit and tendril extracts (data not shown).

The effect of bitter melon leaf extracts on the Pgp-mediated efflux of vinblastine was examined. As in the [^{3}H]-vinblastine accumulation experiments, the leaf extracts caused a dose-dependent decrease in the amount of [^{3}H]-vinblastine efflux from cells and resulted in an increase in intracellular [^{3}H]-vinblastine retention. The leaf extracts at concentrations of 50, 75, 100 µg/ml enhanced vinblastine retention by approximately threefold, fivefold, and sixfold, respectively (Fig. 3b). In comparison, verapamil at 20 µ*M* enhanced vinblastine retention fourfold, demonstrating that the leaf extracts at concentrations of 75 and 100 µg/ml inhibited drug efflux more potently than 20 µ*M* of verapamil (Fig. 3b). The leaf extracts increased [^{3}H]-vinblastine retention in KB-V1 cells but not in KB-3-1 cells which lack Pgp (Fig. 3b).

Discussion

The presence of Pgp has been demonstrated using monoclonal antibodies or gene probes [3, 4, 18]. Drug-resistant KB-V1 cells have been shown to express only Pgp at high levels [22] on their plasma membrane, but Pgp is not expressed in the drug-sensitive KB-3-1 cells. As KB-3-1 and KB-V1 cell lines have been characterized extensively with respect to the phenomenon of MDR as well as the function of Pgp, we decided to use these cell lines to assess the effect of bitter melon extracts on the function of Pgp and reversal of the MDR phenotype.

The intracellular levels of many chemotherapeutic drugs are decreased by the activity of Pgp, which are upregulated in many drug-resistant cancer cells [14, 21] Therefore, the development of agents which inhibit the Pgp-mediated efflux of drugs, and thus reverse MDR, has been intensively pursued. Verapamil and cyclosporin A are the most effective Pgp inhibitors in vitro; unfortunately, they have limited clinical use. Clinical use has been disappointing due to dose-limiting toxicity and other side effects, i.e., verapamil is a calcium channel blocker and cyclosporin A is immunosuppressive. Nontoxic Pgp inhibitors derived from plant-based chemicals may prove to be efficacious when administered in combination with commonly used chemotherapeutic pleiotropic drugs such as vinblastine. Bitter melon is one of the important Thai traditional medicines in cancer treatment, but its effects at the cellular level remain unknown. We were especially interested in its effects on drug-resistant cells, as resistance is a major obstacle to the success of cancer chemotherapy and has been closely associated with treatment failure.

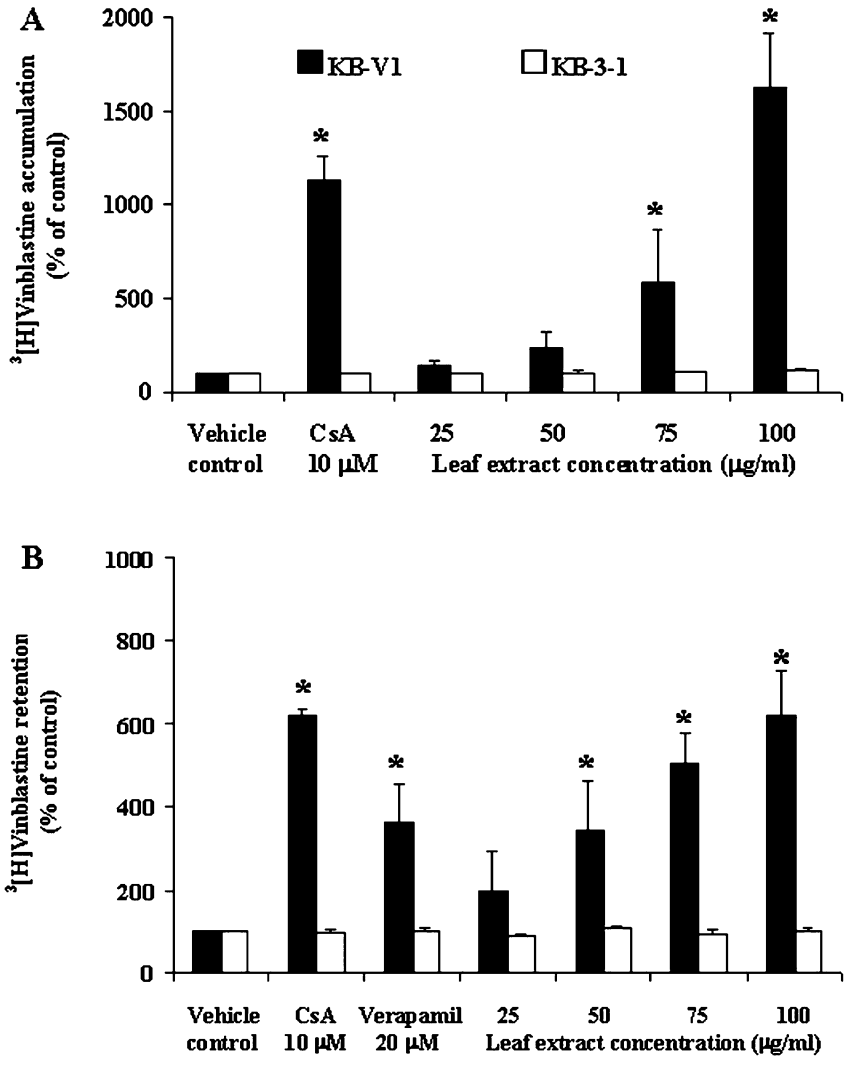


Fig. 3a, b Effect of bitter melon leaf extract on (**a**) [^{3}H]-vinblastine accumulation and (**b**) efflux in KB-V1 and KB-3-1 cell lines. The amount of intracellular radioactivity in the presence of the positive controls cyclosporin A (CsA) and verapamil and various concentrations of the leaf extract were determined using a β counter. Each point represents the mean value of three independent experiments performed in duplicate. *$P < 0.05$ vs vehicle control

In the present study we investigated the effects of bitter melon extracts from leaves, fruits and tendrils on drug accumulation and Pgp activity in vitro. The leaf extract showed a concentration-dependent effect on Pgp-mediated vinblastine accumulation and efflux in drug resistant KB-V1 cells, but had no effect in drug-sensitive KB-3-1 cells which lack Pgp. There was no change in drug accumulation and efflux in KB-V1 cells in the presence of fruit and tendril extracts. Since the time of exposure of cells to the bitter melon extracts in Pgp functional assays was short (1–2 h), it is unlikely that the extracts acted by downregulating MDR1 transcription which would have reduced the amount of cellular Pgp. Nevertheless, the protein levels of Pgp were determined and the results showed that Pgp expression in KB-V1 cells was not affected by treating the cells with bitter melon leaf extract (data not shown). Vinblastine is known to be a good substrate for Pgp, and we conclude that bitter melon leaf extract modulates intracellular drug levels by inhibiting Pgp activity.

Treatment of drug-resistant cells (KB-V1 cells) with bitter melon leaf extract increased the sensitivity of those cells to vinblastine, but similar treatment of KB-3-1 cells showed no modulating effect. The fruit and tendril extracts did not affect the MDR phenotype in either cell line. In summary, the leaf extracts from bitter melon showed reversal effects on the MDR phenotype which is consistent with increased intracellular accumulation of the drug. Although the present study demonstrated that bitter melon leaf extract is an effective inhibitor of Pgp activity in vitro, animal experimentation is required to determine if the bitter melon leaf extract has potential as an effective chemosensitizer for treating cancers expressing Pgp-mediated MDR.

The exact nature of active components of bitter melon leaf extract remains to be identified. Additional experiments are required to: (1) identify the active compound(s) present in the bitter melon leaf extracts, and (2) explore the effect of bitter melon extract on other drug efflux mechanisms such as the MDR-related proteins [7, 8, 15, 16, 20].

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