Evaluation of the potential effect of Allium sativum, Momordica charantia, Eugenia jambolana, Ocimum sanctum, and Psidium guajava on intestinal p-glycoprotein in rats

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ABSTRACT
Aims/Background: This study was evaluated synergistic effect of a polyherbal formulation (PHF) of Allium sativum L., Eugenia jambolana Lam., Momordica charantia L., Ocimum sanctum Linn., and Psidium guajava L. on p-glycoprotein (Pgp) of intestine. These five herbs were traditionally used for diabetes. These herbs are commonly present in Ayurvedic product as antidiabetics in India. Materials and Methods: PHF was prepared by five indigenous herbs. Different doses (50, 100 and 200 mg/kg/day) were orally administered to Sprague-Dawley rats of different groups for multiple weeks except control groups. Alteration in Pgp expression was evaluated by real-time-polymerase chain reaction and western blotting while modulation in activity of Pgp was evaluated using rhodamine 123 (Rh123) as transport substrate by in-situ absorption and everted gut sac method. Results: In PHF, pretreated group received 50, 100 and 200 mg/kg/day for 7 days, mRNA level decreased by 1.75, 2.45 and 2.37-fold, respectively, as compared to control. Similarly, when PHF at dose of 100 mg/kg/day was given consequently for 4 weeks, maximum decrease in Pgp expression level was observed only after 1 week and further increase in the treatment duration did not produce significant decrease compared to the 1st week treatment. Pgp mediated transport of Rh123 was significantly decreased with everted gut sac prepared from PHF pretreated rats (1 week) compared to those prepared from vehicle treated rats. Conclusions: We report that PHF pretreatment downregulated the expression of intestinal Pgp and this downregulated intestinal Pgp would result in decreased functional activity. In addition, this downregulated Pgp expression might affect the bioavailability of antidiabetic Pgp substrate drugs.

KEY WORDS: Everted-sac, herb-drug interaction, polymerase chain reaction, p-glycoprotein, western blot

INTRODUCTION
Diabetes mellitus becomes a growing problem in the contemporary world. It is the 7th foremost cause of death in the world. Approximately, 1.5 million deaths occurred in 2014 due to the Type 2 diabetes mellitus. This is a more common in developing countries. There were 366 million people suffering with Type 2 diabetes in 2011; these numbers will rise to 552 million by 2030 [1]. It is one of the intractable diseases identified by Indian council of medical research for which an alternative medicine is need for the treatment herbal remedies are safe and fascinating move toward alternative medicine for diabetes complementary medicine because their benefits are well documented from a historical viewpoint in the population of diabetes [2]. Most of the diabetics in India rely on the use of conventional medicines for the major health care needs [3]. However, the consumption of herbal preparation has been reported for their induction and inhibition potential on p-glycoprotein (Pgp) expression [4]. Pgp is an ATP-dependent multidrug efflux transporter and an absorption
barrier by transporting some drug from intestinal cells from the lumen [5,6]. Pgp is considered as one of the major barriers for bioavailability of orally administered drugs. Therefore, any alteration in expression and activity of Pgp will eventually result in increase or decrease in plasma concentration of Pgp substrate drug. Such herb-drug interaction may be hazardous if Pgp substrate drug levels reaching above toxicity thresholds or may change drug absorption, distribution, and elimination. Among various transporter proteins, Pgp is a major efflux protein that actively efflux the Pgp substrate in intestinal lumen.

Several compounds derived from the natural product and plant extracts have been reported to inhibit Pgp activity [7,8]. In this study, Allium sativum L., Eugenia jambolana Lam., Momordica charantia L., Ocimum sanctum Linn., and Psidium guajava L., which have been commonly used for the treatment of diabetes or consumed daily in Indian population [9-14], were monitored for their synergistic effect on intestinal Pgp efflux transporter. All these plants were used in the polyherbal formulation (PHF). The PHF has shown a significant antidiabetic effect on streptozotocin in induced diabetic rats (data are not shown in the manuscript). Here, we have estimated the effect of PHF on altering intestinal Pgp expression and its function. Alteration in Pgp expression was done using western blotting while modulation in the activity of Pgp was evaluated using rhodamine 123 (Rh123) transport study. To achieve these aims, we studied the effect of PHF administration on transcriptional level of Pgp and its functional activity in dose and time dependent manner.

The previous studies are only based on a single herbal active constituent. However, in Ayurvedic system of medicine practice, a combination of polyherbal preparations are prescribed but no such studies are documented in literature. Moreover, these formulations are also not told to prescribing physician by the patients. This may due to the ignorance of patients because these polyherbal preparations are not considered as a part of active medication. Prolong usage of these formulations may alter the expression of efflux transporters that finally leads to altered the bioavailability of antidiabetic or other Pgp substrate drugs that have narrow therapeutic index.

MATERIALS AND METHODS

Reagents and Chemicals

Phenylmethanesulfonyl fluoride (PMSF), Rh123, bovine serum albumin (BSA), protease inhibitor cocktail, Dulbecco’s phosphate buffered saline (PBS), sodium dodecyl sulfate (SDS), sodium pentobarbital, Triton-X, TEMED, acryl and bis-acrylamide, ammonium persulfate and Evans blue were purchased from Sigma-Aldrich (USA). High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were purchased from Sigma-Aldrich (St. Louis, MI, USA).

Preparation of PHF

Five herbs, A. sativum L. (garlic), E. jambolana Lam. (Jamun) seeds, M. charantia L. (Bitter gourd) fruits, O. sanctum Linn. (Holy Basil) leaves, and P. guajava L. (guava) were purchased from the local vegetable market from the Lucknow, Uttar Pradesh, India. PHF was prepared by mixing 200 mg powder of each herb in single formulation. First, the selected herb materials were shade dried and grinded by mixer grinder. Prepared hydro-alcohol extracts of herbs were concentrated using rotary evaporator at 40°C temperature than extracts were freeze-dried at −20°C for 12 h afterward lyophilized using lyophilizer. The lyophilized extracted powders were placed in an airtight glass box and kept in the desiccator until used.

Animals

The animal experimental procedures were carried out in accordance with current legislation on animal experiments as per Institutional Animal Ethical Committee at King George Medical University, Lucknow (IAEC approval no IAEC/2013/4). Male Sprague-Dawley (SD) rats of weight between 220 ± 20 g were purchased from CSIR-IITR (India). Animals were maintained at 25°C temperature in steel cages with alternate 12 h of light and dark cycles and given a pellet diet and water. Before starting the experiment rats were acclimatized for 7 days then divided into two groups, PHF pretreated (n = 5), and vehicle treated (n = 5). Rats in the pretreated group were administered orally with PHF (50, 100, and 200 mg/kg/day) 16-gauge gavage needle for 7 days and multiples of weeks accordingly. The PHF suspension was made in 0.5% sodium carboxymethyl cellulose for oral administration. The control group received the same volume of the vehicle for 7 days. Animals were allowed free access to food and water but before euthanasia, rats were overnight fasted to decrease the intestinal content. At the end of the experiment, rats were sacrificed by inhalation of anesthetic ether.

Dose Response and Time Dependent Experiment

To evaluate the effect of dose response of PHF on intestinal Pgp of rats were given 50, 100, and 200 mg/kg/day by oral administration for 7 days and multiple weeks. The control group received same volume of the vehicle for 7 days. During time-dependent, course of experimentation rats were given 100 mg/kg/day of PHF consequently for 7, 14, 21 and 28 days [15].

Effect of PHF Pretreatment on Gastrointestinal Pgp Expression and Activity

The effect of PHF administration on intestinal Pgp was evaluated at transcriptional and translational level. Similarly, the effect of PHF administration on intestinal Pgp activity was evaluated using everted gut sac methodology.

Real-time Polymerase Chain Reaction (RT-PCR)

mRNA isolation, cDNA synthesis, primer designing was performed as Singh et al. The forward and reverse primer sequence for ATP-binding cassette subfamily B member 1 (ABCB1) is 5’-TGATGCTTTCGCCAAATGC-3’ and 5’-TGTCCTCTCTCTGAAAAACTGTC-3’, respectively. Glyceraldehyde
3-phosphate dehydrogenase (GAPDH) was used as an internal loading control and sequence is 5’-AGCTGTTCTCATCATGTTGAA-3’ and 5’-ATTTGATGTTAGGCGGATCG-3’, respectively. Effect of pre-treatment of PHF on the mRNA expression of Pgp was examined by RT-PCR. For quantitative RT-PCR, cDNA was amplified using Light Cycler 480 (Roche Molecular Biochemicals, USA) using SYBR green kit (Fermentas, USA). The reaction was performed in 96-well white PCR plate. The reaction mixture was consisted of 1X SYBR green, 200-300 nM primers and 2 µl of cDNA in final reaction volume of 20 µl. The thermal cycle condition was 95°C for 15 min to activate Amplitaq Gold DNA polymerase, 95°C for 15 s and anneal/extension at 60°C for 1 min (40 cycles). GAPDH was used as an internal loading control. Fold changes in mRNA level are derived after normalizing with GAPDH mRNA level [6,15].

Preparation of Crude Intestinal Membrane Fractions

Crude membrane fractions were prepared from the intestinal mucosa after every week in 28 days of successive PHF treated and control rats [16]. Intestine was immediately excised out and washed with ice-cold normal saline containing 1 mM PMSF as a protease inhibitor then snap frozen and stored at −80°C until analysis. Intestinal samples were homogenized in the 50 mM tris buffer (pH 8) containing 1% Triton-X-100, 1 mM PMSF and cocktail protease inhibitor (Sigma-Aldrich, USA). Centrifugation of homogenate was performed at 10,000 rpm for 20 min at 4°C. Protein in the supernatant was examined using the Lowry method with BSA as standard [17].

SDS-Polyacrylamide Gel (PAGE) and Western Blotting

About 50 µg of crude membrane fraction was boiled for 10 min in denaturing sample buffer (1% SDS, 1% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue, 10 mM Tris-HCl; pH 6.8). In the crude intestinal membrane fractions, protein was separated on 8% SDS-PAGE. Resolute proteins were electrophoretically moved to polyvinylidene difluoride membrane (Fermentas, USA). Membrane was blocked for nonspecific sites in phosphate buffer (containing 0.05% Tween 20 and 5% skimmed milk) for 2 h and washed 2 times in PBS tween (PBST) for 5 min then probed with antibodies for Pgp (1:700; Santa Cruz Biotechnology Dedham MA), and then re-probed with β-actin antibody (1:5000; Cayman Chemical Company, Michigan, USA) for loading correction. Then, the blots were washed three times in PBST with 0.1% Tween 20 and incubated with 1:10,000 dilution of secondary antibody (anti-immunoglobulin G-horseradish peroxidase conjugate) for 2 h at room temperature. After extensive washing in PBS, substrate solution was applied to the membrane, then incubation was carried out for 5 min at room temperature. Bands were examined and visualized on X-ray film with enhanced chemiluminescence western blot kit (ECL, Millipore), following the manufacturer’s protocol.

In-vitro Transport of Pgp Substrate Rh123 across PHF Treated Rat Intestine

In-vitro transport of Rh123 across rat everted gut sacs was performed [18]. Briefly, at the end of pre-treatment of PHF, rats were sacrificed, and ileum was excised out, then washed with normal saline, and everted using a glass rod. Small intestine was segmented into 4-5 cm long segments that were tied at one end. The Rh123 solution (25 µM in TC199) was filled in the serosal side and tightly ligated to make a gut sac. Immediately, this intestinal everted sac was placed in 30 ml of TC 199 medium. The solution was gassed by 5% CO2/95% O2 and maintained at 37°C during the experiment. Transport of the Rh123 across the serosal to the mucosal side was calculated by sampling 1 ml of the external medium every 10 min up to 50 min. The rate of Rh123 transport was expressed as µM or percentage secreted per minute in the mucosal compartment. In some experiments, Pgp inhibitor (verapamil 100 µM) was added in the mucosal and the serosal sides at the same concentration. This model was validated with animals pre-treated with dexamethasone (100 mg/kg/day for 2 days) by oral route [19].

Assay Method for Rh123

The determination of Rh123 concentrations in the samples was performed immediately after the experiment. For plasma samples, an equal volume of methanol was added to 100 µl aliquots of plasma to precipitate proteins. The mixture was vortexed for 2 min and centrifuged at 9000 x g for 10 min at 4°C and supernatant thus obtained was used for analysis. However, the perfusate and samples from everted sac were directly used. 100 µl aliquots of the samples (supernatant or perfusate and everted sac samples) were then added to 96-well black plates, and readings were taken at an excitation wavelength of 485 nm and emission wavelength of 527 nm on multimode microplate reader (Tecan, USA). Rh123 concentration in the samples was derived from standard curve of Rh123.

Excretion Study of Rh123 Using In Situ Perfusion Method

Total two group of animals, viz., control (5 rats) and two PHF treated (5 rats) were prepared. Each group of rats was fasted overnight for at least 12 h and on the 8th day, animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg). They were positioned in a supine position on a heating pad to maintain constant normal body temperature under a surgical lamp. The upper jejunum (15 cm) of the intestinal lumen was catheterized with an inlet silicon tube (4.2 mm i.d.). This was perfused with PBS containing 25 mM glucose into the intestinal lumen in a single perfusion manner at a flow rate of 0.5 ml/min [18]. After 30 min of perfusion for stabilization, 0.2 mg/kg of Rh123 was injected via the femoral vein. The intestinal perfusate was collected every 15, 30, 45, 60, 75, 90, 105 and 120 min. Samples were stored at 4°C after collection. Rh123 excreted at each time point and cumulative amount of Rh123 was determined and used as function of Pgp activity.

Statistical Analyses

All data were presented as mean with standard error. Statistical analyses of data were performed using ANOVA and if applicable followed by the Student Newman–Keuls multiple range tests.
Statistical analysis of data was performed using t-test followed by unpaired test. The level of significance was set a priori at \( P > 0.05 \).

RESULTS

Effect of PHF Pretreatment on Gastrointestinal Pgp Expression

The ABCB1 mRNA expression profile of intestine of PHF pre-treated and the vehicle pre-treated group was compared. In PHF pre-treated group that received 50, 100 and 200 mg/kg/day for 7 days mRNA level decreased by 1.75 (\( P < 0.05 \)), 2.45 (\( P < 0.05 \)) and 2.37 (\( P < 0.05 \)) fold respectively as compared to vehicle pre-treated group. Similarly, when PHF at dose of 100 mg/kg/day was given accordingly for 4 weeks maximum decrease in ABCB1 expression level was observed only after 1 week and further increase in the treatment duration did not decrease significantly compared to the 1\textsuperscript{st} week treatment [Figure 1].

Western blotting was carried out for approving the mRNA data with protein level. Results showed the decreased intensity of bands from PHF treated rats as compared to control rats. Intensity of the bands revealed that Pgp expression has been downregulated with PHF treatment [Figure 2].

Effect of PHF Pretreatment on Intestinal Pgp Activity

In-vitro everted gut sac methodology was first validated using a known inducer of Pgp expression. The measurement of Rh123 transport was calculated in the absence and presence of Pgp inhibitors in everted gut sac prepared from PHF pre-treated and control rats. In absence of verapamil, the transport of Rh123 was decreased by 55 (\( P < 0.01 \)) and 60% (\( P < 0.01 \)) at 100 and 200 mg/kg/day of PHF for 7 days compared with control rats [Figure 3]. On the other hands, no significant alteration was observed at 50 mg/kg/day of PHF dose.

However in the presence of verapamil, Rh123 transport across the everted gut sacs was significantly decreased by 59% (\( P < 0.05 \)), 61% (\( P < 0.05 \)) and 73% in control, 100 and 200 mg/kg/day of PHF when administered for 7 days, respectively [Figure 3]. The Pgp-mediated transport of Rh123 was significantly decreased by 62% (\( P < 0.05 \)) and 72% (\( P < 0.01 \)) at 100 and 200 mg/kg/day of PHF dose for 7 days, respectively, compared with the control group [Figure 3].

In the time-dependent study evaluation of the effect of treatment duration, PHF (100 mg/kg/day) was administered orally for 4 weeks and Rh123 transport across everted gut sac was evaluated after every week. Basal transport of Rh123 (without inhibitor) was significantly (\( P < 0.01 \)) decreased by 52%, 55%, 58% and 56% after every week of PHF treatment, respectively, up to 4 weeks in comparison to control. No significant difference was obtained between different treated and control groups.

Similarly, in the presence of verapamil, Rh123 transport across the everted gut sac was considerably decreased by 47%, 51%, 49% and 49% after every week of PHF treatment, respectively, up to 4 weeks in comparison to control. The Pgp mediated transport of Rh123 was significantly (\( P < 0.01 \)) decreased by 63, 65, 68 and 70% after every week of PHF treatment, respectively, up to 4 weeks in comparison to control [Figure 4].

In excorption study with Rh123, PHF (100 mg/kg/day) and vehicle were orally administrated in treated group and control group, respectively for 7 days and excetration of Rh123 from intestinal lumen was calculated after designated time interval. It was observed that intestinal lumen excretion of Rh123 was decreased at each time point in PHF treated rats as compared to control. Similarly, the cumulative excretion of Rh123 after 120 min was significantly decreased by 34% (\( P < 0.05 \)) in PHF treated rats in comparison to control and this excreted amount of Rh123 over 120 min in intestine of control, and PHF treated rats was 0.72 ± 0.02 and 0.46 ± 0.02 µg/mL, respectively [Figure 5].

DISCUSSION

Intestinal Pgp is the major efflux transporter that confines the bioavailability of orally administered drugs. Any alteration in level and/or activity of this protein would ultimately result in the altered pharmacokinetics of substrate drug. Among various factors, consumption of herbal preparation is a major factor that can modulate the expression and/or activity of Pgp. Various herbal preparations have been reported to alter the expression and activity level of this protein, e.g., St. John Wort consumption increased the expression of Pgp that eventually decreased the bioavailability of cyclosporin resulting in rejection of tissue implant in the patient. Other clinical drug-drug and herb-drug interactions were reported in the literature between digoxin (Pgp substrate) and other Pgp substrates such as quinidine, verapamil, talinolol, clarithromycin, traconazole, erythromycin, and propafenone [20]. Herbs in PHF that has been used from ancient times to improve glycemic control. However, no reports are available regarding the synergistic effect of this type formulation on Pgp. Therefore, in this study, we evaluated in-vivo effect of PHF administration in dose and time.
dependent manner in male SD rats. One possible method of estimating the induction/inhibitory potency of any compound to protein is measuring mRNA and protein levels. However, the relationship between mRNA and protein levels has not been established yet, and using mRNA and protein levels alone may misleading to the contribution of Pgp on the pharmacokinetics of drugs. Assessing functional activity for induction/inhibition potential of any compound is supposed to be best because it is only the functional activity that comes to play the following treatment. However, in this study, we used all three methods to analyze the induction/inhibition potential of PHF. In our data, we obtained a decrease in mRNA and protein level of Pgp in intestine of rats with PHF treatment. The reason behind this phenomenon might be attributed to the overall exposure of intestine, which is more exposed to oral components compared to liver, kidney, and other parts of the body. Therefore, intestinal

Figure 2: Dose and time-dependent effect of PHF administration on intestinal Pgp protein level. In dose response experiment rats were orally gavaged with 50, 100 and 200 mg/kg/day of PHF extract for 1 week. In time response study rats were gavaged with PHF (100 mg/kg/day) consecutively for 28 days and any alteration in mRNA expression was assessed after every 7 days. T1, T2, T3 and T4 represent treatment for 7, 14, 21 and 28 days, respectively. Control group received the same volume of blank formulation for same time duration.

Figure 3: Dose response of PHF administration on intestinal Pgp activity. Rh123 transport was performed in absence and presence of inhibitor (verapamil 100 µM) across the everted gut sac obtained from control and PHF treated rats. Each value (percent of Rh123 transported per min) is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (* P<0.05 and ** P<0.01) from control.

Figure 4: Time dependent effect of PHF administration on intestinal Pgp activity. Rh123 transport was performed in absence and presence of inhibitor (verapamil 100 µM) across the everted gut sac obtained from control and PHF treated rats. C, T1, T2, T3 and T4 represents control group, one, two, three and four week treated group, respectively. Values are expressed as percent of Rh123 transported per min and are mean ± standard deviation of three replicates.
Pgp is considered a more important in determining the oral bioavailability of Pgp substrate drugs.

There is a not true measure of functional activity of Pgp either increase or decrease in mRNA and protein; we measure the functional activity both in-vivo and in-vitro following the PHF pretreatment using Rh123, as a typical Pgp substrate. The fluorescent dye Rh123 has been extensively used as index of Pgp-mediated transport in rodents and various tissue culture models [21]. Tian et al. (2002) reported that the measurements of Rh123 using a spectrofluorometer were as same as those using HPLC method in a transport study using the rat everted-sac method [4]. Therefore, the use of spectrofluorometer in our study as a convenient tool can be rationalized.

Rh123 has been reported to be metabolized by intestinal esterase and some of its metabolite also has fluorescence intensities [4]. However, it has been considered that intestinal esterase during the study had lesser effect on the fluorescence intensity of Rh123 in everted gut sac method because intestine was washed several times with normal saline before use and everted sac is in direct contact with only Rh123. Similarly, in-situ perfusion method also has much less effect on intestinal esterase activity because intestinal lumen was constantly perfused with buffer solution and samples were immediately analyzed.

In our study, Pgp mediated transport of Rh123 was significantly decreased with everted gut sac prepared from PHF pretreated rats (1-week pretreatment) compared to those prepared from vehicle treated rats. However, this transport inhibition due to the presence of PHF components in intestine was denied because the experiment was conducted after the 24 h of last PHF dose with fasting of rats for 8-12 h. This duration is because the experiment was conducted after the 24 h of last PHF treatment using RH123, as a typical Pgp substrate. The fluorescent dye RH123 has been extensively used as index of Pgp-mediated transport in rodents and various tissue culture models [21]. Tian et al. (2002) reported that the measurements of RH123 using a spectrofluorometer were as same as those using HPLC method in a transport study using the rat everted-sac method [4]. Therefore, the use of spectrofluorometer in our study as a convenient tool can be rationalized.

To validate this hypothesis, we performed in-vivo functional activity assay (exsorption of RH123). The result obtained in this experiment revealed that PHF pretreatment decreased the exsorption of RH123 into intestinal lumen in comparison to control. These data support our hypothesis that increase in tissue concentration of Pgp substrates was due to downregulation in expression and activity of intestinal Pgp. In addition, RH123 is not a CYP3A substrate, therefore, the modulation in distribution of RH123 was attributed to decrease in Pgp expression only. These in-vivo results were in good agreement with the results, we obtained in western blot and ex-vivo everted gut sac. Therefore, the results obtained could be significantly attributed to downregulation of intestinal Pgp. Alteration in the expression and activity of Pgp with herbal constituents could also be a useful strategy to increase the oral bioavailability of Pgp substrate, in particular, to develop the oral formulations of antidiabetic drugs such as sitagliptin, glibenclamide, rosiglitazone and troglitazone, transported by Pgp transporter [22]. However, further investigations to estimate quantitatively Pgp in human cell line like Caco-2, intestinal epithelial as well as pharmacokinetic studies with Pgp substrates are required to validate their effect to clinical implementation.

**CONCLUSION**

This study demonstrates that PHF pretreatment downregulated the expression of intestinal Pgp, and this downregulated intestinal Pgp would result in decreased functional activity. Based on these results, PHF inhibits the efflux activity of
Pgp transporter which could play a key role in modulating the systemic availability of Pgp substrate drugs in vivo. It has been reported in literature that regular consumption of herbal preparation could alter the expression of Pgp in intestine and other tissues. In addition, this downregulated Pgp expression might affect the bioavailability of antidiabetic Pgp substrate drugs.

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AUTHORS’ CONTRIBUTIONS

Experiment is conceived and designed by DK and RKD. DK and NT performed experiments and participated in data collection, interpretation and statistical analysis. DK wrote the paper.

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