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Evaluation of herb-drug interaction of a polyherbal Ayurvedic formulation through high throughput cytochrome P450 enzyme inhibition assay

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ABSTRACT

Ethnopharmacological relevance: Arishtas are Ayurvedic formulation made with decoction of herbs. Arjunarishta formulation is being used in Ayurveda for cardio-protective activity. Ashwagandharishta formulation possesses antioxidant, anti-atherosclerotic and anti-stress properties. Ridayarishta, a novel empirical formulation was prepared using combination of selected ingredients from these two formulations to support healthy heart functions and to reduce stress.

Aim of the Study: Aim of the Study was to investigate herb-drug interaction (HDI) of Ridayarishta formulation through human hepatic cytochrome P450 (CYP450) enzyme inhibition assay.

Materials and methods: Ridayarishta formulation was phyto-chemically standardized against arjunolic acid, arjunetin, berberine, piperine, resveratrol and withaferin-A using high performance thin layer chromatography (HPTLC) analysis. The formulation was standardized with respect to ethanol by gas chromatographic (GC) analysis. HDI was evaluated with Ridayarishta formulation and amlodipine besilate, atenolol, atorvastatin, metformin, glipizide glimepiride cocktail using high throughput CYP450 enzyme inhibition assay; against CYP1A2, 2C19, 2D6 and 3A4 isozymes.

Results: Contents of arjunolic acid, arjunetin, berberine, piperine, resveratrol and withaferin-A in Ridayarishta formulation were found to be 1.76 ± 0.12, 1.51 ± 0.09, 1.85 ± 0.05, 3.2 ± 0.12, 1.21 ± 0.08, and 2.16 ± 0.09 ppm, respectively. Quantity of ethanol in Ridayarishta was found to be $7.95 \pm 0.023\%$ (V/V). Ridayarishta showed significantly higher ($P < 0.001$) IC_{50} value against CYP1A2 (IC_{50}–13.80 ± 1.96 μg/mL), 2C19 (IC_{50}–14.343 ± 2.28 μg/mL), 2D6 (IC_{50}–0.897 ± 0.28 μg/mL) and 3A4 (IC_{50}–32.057 ± 2.51 μg/mL) compared to positive controls such as furafylline, tranylcypromine, quinidine and ketoconazole respectively. Cocktail of herbal formulation and cardio protective, antihypertensive, anti-diabetic drugs showed significantly ($P < 0.001$and $P < 0.01$) less or negligible HDI.

Conclusion: Ridayarishta formulation alone and cocktail with amlodipine besilate, atenolol, atorvastatin, metformin, glipizide, glimepiride had negligible or insignificant effect on CYP450 inhibition. It may be concluded that consumption of Ridayarishta along with selective cardio protective, antihypertensive and anti-diabetic conventional medicine is safe with negligible or without any significant CYP450 (CYP1A2, 2C19, 2D6 and 3A4) inhibition mediated HDI.

1. Introduction

Arishtas are classical Ayurvedic formulation made with decoction of herbs. These unique liquid dosage forms contain self-generated alcohol which improves the efficacy of extraction of alcohol soluble molecules along with water soluble molecules from the herbs; to enhance drug delivery into human body site (Sekar and Mariappan, 2008). Arjunarishta is an Arishta formulation supports normal cardiac functions, loss of appetite and immune system traditionally (The Ayurvedic formulary of India, 2003). Ashwagandharishta formulation is being used in Ayurveda to relieve stress, reduces the chances of cardiovascular risk factors and mental disorders (The Ayurvedic formulary of India, 2003;

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Tiwari and Patel, 2010). Important precursors of Arjunarishta and Ashwagandharishta were selected based on Ayurvedic literature and blend them to get a proprietary formulation for two associated indications. Ridayarishta, an empirical novel formulation having cardio protective and stress reliever properties was developed by decoction of 25 major Ayurvedic plants (Supplementary Table S1), followed by self-fermentation process. Principal ingredients of Arjunarishta having cardioprotective properties and Ashwagandharishta having anti-stress activity were selected for preparation of this formulation.

Standardization parameter for Ayurvedic medicine with respect to bioactive compounds is vital to maintain quality control and batch to batch reproducibility (Pandit et al., 2011a). Standardization of Ayurvedic Arishtas formulation with respect to ethanol is foremost important to maintain its quality and efficacy. Among these herbs *Terminalia arjuna* W. and A., *Vitis vinifera* Linn. and *Withania somnifera* Dunal. were used in higher quantity. Arjunolic acid of *T. arjuna* is proven for prevention of myocardial necrosis, platelet aggregation and lowering of blood pressure, heart rate and cholesterol levels (Hemalatha et al., 2010). Cardio protective and antimicrobial activities of arjunetin have also been reported (Aneja et al., 2012). Cardio protective effect of *V. vinifera* derived polyphenol resveratrol has been well proven (Wu et al., 2011). Withaferin A, an active compound from *W. somnifera*, has been endorsed its usefulness for anti-inflammatory, cardio protective and vascular inflammatory diseases (Lee et al., 2012). Principle marker compounds such as Arjunolic acid, arjunitin, berberine (*Berberis aristata*), piperine (*Piper longum and Piper nigrum*), resveratrol and withaferin-A of these plants were selected in order to phytochemical standardization of Ridayarishta formulation.

Herb-drug interaction (HDI) study is the key marker for determination of adverse drug reaction (ADR) of herbal medicine and conventional pharmaceuticals. Among the Cytochrome P450 (CYP450) superfamily, 1, 2 and 3 are majorly responsible for xenobiotic and drug metabolism in human liver (Anzenbacher and Anzenbacherova, 2001). 5 human CYP isoforms (CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4) are responsible for 80% of the drug metabolism (Arora et al., 2015). CYP450 inhibition or induction is the most commonly studied mechanism for the HDI (Mukherjee et al., 2011; Shivaprasad et al., 2014). Intestinal and hepatic CYP450 is responsible for metabolism of numerous structurally unrelated compounds. Multi-drug combination therapy is now a common practice for numbers of diseases and the interaction between herbal and conventional drugs are inescapable (Mukherjee et al., 2011; Pandit et al., 2012). Thus, it is prime important to evaluate hepatic CYP450 mediated HDI of Ridayarishta formulation to get knowledge about any ADR. Based on above context, CYP1A2, 2C19, 2D6 and 3A4 inhibition mediated HDI of Ridayarishta was studied with cocktail of selective cardioprotective (atenolol), anti-hypertensive (amlodipine besilate, atorvastatin) and anti-diabetic (metformin, glipizide and glimepiride) drugs. High-performance thin layer chromatography (HPTLC) was used in order to standardize the Ridayarishta formulation against arjunolic acid, arjunetin, resveratrol and withaferin-A. Moreover, gas chromatographic (GC) analysis was also carried out for standardization of Ridayarishta formulation with respect to ethanol.

2. Material and methods

2.1. Chemicals

CYP1A2 (Catalogue no.: 459500), CYP2C19 (Catalogue no.: 459400), CYP2D6 (Catalogue no.: 459200) and CYP3A4 (Catalogue no.: 459100) high throughput inhibitor screening kit were procure from CORNING (Discovery labware, Inc., Woburn, MA, U.S). Kit components contains cDNA expressed recombinant human CYP1A2 (Cat. No. : HTS-703) using baculovirus infected insect calls, potassium phosphate (pH 7.4) buffer; Tris base, $NADP^+$, $MgCl_2$, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, CEC (3-Cyano-7-Ethoxycoumarin), Furafylline, CHC (3-cyano-7-hydroxycoumarin); CYP2C19 (Cat. No. : 04-80759), CEC, Tranylcypromine, CHC; CYP2D6 (Cat. No.: 04-80717), AMMC (3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarine), quinidine and AHMC (3-[2-(N,N-diethylamino) ethyl]-7-hydroxy-4-methylcoumarine hydrochloride); ketoconazole, BFC (7-benzyloxy-trifluromethylcoumarin); HFC (7-hydroxy-trifluromethylcoumarin). Fluorimetric screening was performed using the 96 well black-microplates (NUNC, Roskilde, Denmark). Tablets of amlodipine besilate (Stamlo), atenolol (Aten), atorvatatin calcium (Atocor), metformin hydrochloride (Glyciphage), glipizide (Glide), glimepiride (Glimulin) were purchased from local vendor. All the solvents and chemicals for standardization and sample preparation were of analytical grade and purchased locally. Arjunolic acid (purity $\geq$95%, LC/MS-ELSD), berberine (purity $\geq$97%, HPLC), piperine (purity $\geq$97%, HPLC), resveratrol (purity $\geq$99%, HPLC) and withaferin-A (purity $\geq$95%, HPLC) were procured from Sigma (Steinheim, Germany). Standard arjunetin (purity $>$95%, HPLC) was obtained from Natural Remedies Pvt Ltd. (Bangalore, India). 0.2 μ Nylon syringe filter was procure from membrane Solution (USA). Silica gel $60F_{254}$ precoated plates for HPTLC analysis and all analytical grade solvents were bought from Merck (Mumbai, India).

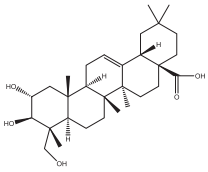
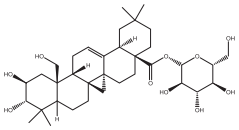
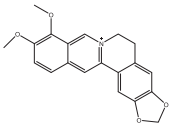
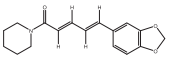
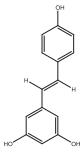
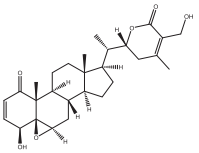
2.2. Ridayarishta formulation

Ridayarishta formulation was obtained from commercial batch (batch No FJ001, manufacturing date: May 03, 2015), prepared at production unit of Emami Limited, VAPI, Gujarat, India. Specimen of the formulation was retained at real time stability study storage chamber at Research & Development Center, Emami Limited, Kolkata, India. List of plants used for preparation of Ridayarishta formulation have been represented in Supplementary Table S1. Plant ingredients used in commercial batch preparation were collected from VAPI unit. Entire plant specimens were authenticated by Mr. Amalesh Nanda, pharmacognosist, Research & Development Center, Emami Limited, Kolkata, India. Voucher specimen (Supplementary Table S1) has been deposited at Research & Development Center, Emami Limited, Kolkata, India for further references.

2.3. Preparation of test samples

Ridayarishta formulation was extracted in chloroform. Solvent was completely removed by rotary evaporator. Extract was reconstitutes in methanol and used for HPTLC analysis. Standard arjunetin (2.5 μg/mL), arjunolic acid (2.5 μg/mL), berberine (1 mg/mL), piperine (0.1 mg/mL), resveratrol (2.5 μg/mL) and withaferin-A (3.0 μg/mL) were prepared in methanol for HPTLC analysis. Samples were filtered through 0.20 μm membrane filter prior to application. Standard ethanol and Ridayarishta formulation was prepared in water and filtered through 0.20 μm membrane filter for GC analysis. Different concentrations ranging from 0.0002 to 2162.7 μg/mL of Ridayarishta was prepared for fluorescence screening assay. Stock amlodipine besilate (25 μg/mL), atenolol (266 μg/mL), atorvastatin (60 μg/mL), metformin (165 μg/mL), glipizide (44.5 μg/mL) and glimepiride (49.2 μg/mL) solutions were prepared in mobile phase consist of acetonitrile and methanol, based on published C_{max} value (Liu et al., 2009, 2010; de Abreu et al., 2003; Zhi-yu et al., 2012, Kobylińska et al., 2000, Jovanović et al., 2006).

Table 1
HPTLC experimental conditions.

Standards	Mobile phase	Densitometry (λ_{max})	Retention factor (R_f) value	Quantities of standards in Ridayarishta formulation (ppm)
 Arjunolic acid	Ethyl acetate:Toluene:Formic acid:Acetic acid=60:30:05:10	690 nm	0.67	1.76 ± 0.12
 Arjunetin		690 nm	0.31	1.51 ± 0.09
 Berberine	n-Butanol:glacial acetic acid: water=12:03:04	366 nm	0.58	1.85 ± 0.05
 Piperine	Toluene:ethyl acetate:di ethyl ether=60:30:10	330 nm	0.24	3.2 ± 0.12
 Resveratrol	Chloroform:Ethyl acetate:Formic acid=25:10:01	307 nm	0.32	1.21 ± 0.08
 Withaferin-A	Chloroform:Methanol=95:05	225 nm	0.37	2.16 ± 0.09

2.4. Standardization of Ridayarishta formulation through HPTLC

Ridayarishta formulation was standardized using HPTLC (CAMAG Muttenz, Switzerland) with respect to major ingredients of the formulation such as arjunolic acid, arjunetin of *T. arjuna* , berberine of *B. aristata,* piperine *of P. longum* and *P. nigrum,* resveratrol of *V. vinifera* and withaferin-A of *W. somnifera*. 5 different spots of the standards and 2 different spots of the Ridayarishta were applied on aluminium-backed HPTLC plates (20 cm × 10 cm) with the help of a CAMAG Linomat V (Muttenz, Switzerland) sample applicator to construct calibration curve. Application rate was 150 nL/s; under constant N_2 gas flow. Spots were applied on 10 mm above the edges with a bandwidth of 8 mm. Ascending development was carried out in a CAMAG twin trough chamber (20 cm × 10 cm), which was pre-saturated with mobile phase. Development was run up to 80 mm of the plate. Developed plate was derivatized with anisaldehyde sulphuric acid reagent for arjunolic acid, arjunetin, berberine and piperine. Plate was scanned using CAMAG TLC Scanner 4 with integrated WinCats software. Detail experimental parameters have been represented in Table 1. Calibration curves were prepared with area under curve of standards (Pandit et al., 2011b). Using this curve quantity of marker compounds in Ridayarishta formulation was determined.

2.5. Standardization of Ridayarishta formulation through GC analysis

Ridayarishta formulation was standardized with respect to ethanol using GC analysis. Analysis was carried out using PerkinElmer Clarus 480 GC system (PerkinElmer, Inc., U.S.A.) consist of FID detector, manual injector and with integrated TotalChrom Navigator-Clarus480 (ver-6.3.2.) software. A Phenomenex ZB Wax Plus capillary Column (60 m × 0.25 mm × 0.25 μm) was used for separation with nitrogen gas (99.999% purity) flowing rate of 1.60 mL/min. 1 μl sample was injected into the column. Injector temperature was set at 200 °C. Oven temperature was programed in the sequence of 5.00 min hold at 50 °C, followed by increased rate of 35 °C/min to 125 °C with hold for 0.5 min, and further increased rate of 30 °C/min to 220 °C with hold for 0.5 min. Duration of each run was 11.30 min. Peak identification in Ridayarishta formulation was achieved by comparing the retention times of reference standard ethanol. Quantity of ethanol in formulation was

estimated by using calibration curve of standard ethanol.

2.6. Fluorometric high-throughput screening (HTS) assays

The assay was performed according to the method described by Pandit et al., (2012) and protocol provided with CORNING kit. Briefly, the entire kit component was subjected to thaw and placed on ice bucket. NADPH-cofactor mixture (1.3 mM $NADP^+$, 66 mM $MgCl_2$ and 66 mM glucose 6-phosphate) was prepared and 144 μL was transferred to the first row of the black micro-well plates. 100 μL cofactor and solvent mixture (methanol, acetonitrile) were added to the remaining wells. 6 μL test sample was added to each row. To calculate the IC_{50} value, three fold serial dilutions were prepared by transferring 50 μL extract and NADPH cofactor mixture from column 1 to column 8. After pre-incubating the plate for 10 min at 37 °C, the reaction was initiated by the addition of 100 μL enzyme–substrate solution. 0.5 M Tris base was used as stop reagent. Fluorescence intensity was measured in a microplate fluorescence reader (BioTek Synergy HT, USA). Percentage inhibition and IC_{50} value were calculated according to the formula described below (Pandit et al., 2011c, 2011d). Detail experimental parameters have been represented in Table 2.

$$\text{Percentage inhibition} = \left[(\text{Control RFU} - \text{Sample RFU})/\text{Control RFU}\right] \times 100$$

RFU = Relative fluorescence units

$$IC_{50} = \left[(50 - LP) \times (HC - LC) + LC\right]/(HP - LP)$$

LP=Low percentage of inhibition; HP=High percentage of inhibition; LC=Low concentration; HC=High concentration.

2.7. Statistical analysis

All the tests were conducted in triplicate. Experimental data were expressed as mean ± SEM. Results were subjected to one way analysis of variance (ANOVA). Statistical significance was calculated using GraphPad InStat Version 5.0. Dunnett's multiple comparison tests was performed by fixing the significance level at $P < 0.05$ and above.

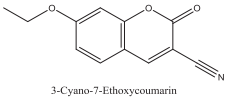
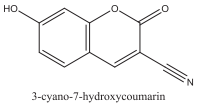
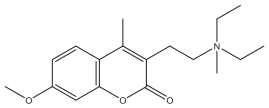
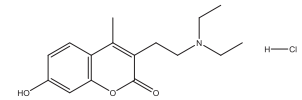
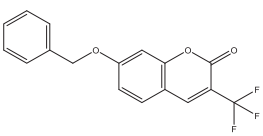
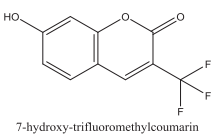
3. Result and discussion

Herbal formulations are being prescribed along with conventional drugs with a purpose of reducing the adverse effects or toxicities associated with conventional pharmaceuticals or to provide synergistic /additive pharmacological effects. The practice of 'polypharmacy' or multi-drug therapy may have resulted serious HDI or drug-drug interactions (Arora et al., 2015). However, numbers of reports have been published signifying that many herbal drugs can cause dramatic alteration in pharmacokinetic properties of the co-administered drugs, affecting their efficacy as well as safety (Mukherjee et al., 2011; Yokotani et al., 2012). Further, medicinal foods are regulated by various laws in different countries and mostly lacking of documentation on their safety profiles, efficacy and quality control. In the present investigation standardization of Ridayarishta formulation was performed by HPTLC and GC analysis. Possible ADR was evaluated with commonly used hypertensive, cholesterol lowering and anti-diabetic drugs through human hepatic CYP450 inhibitory activity studies.

3.1. Standardization of Ridayarishta formulation through HPTLC

HPTLC standardization is a cost-effective, simple and highly selective tool that can ensure quality and batch to batch reproducibility in herbal industry (Pandit et al., 2011b). Presence of marker compounds in Ridayarishta formulation was identified by comparing the R_f values through HPTLC analysis. Calibration curves were linear with a correlation coefficient (r) of 0.99 for all the standards. Calibration curve indicated a good linear dependence of peak area of standards. HPTLC chromatograms of arjunolic acid, arjunetin, berberine, piperine, resveratrol, withaferin-A and Ridayarishta formulation in different solvent systems have been represented in Supplementary Figs. S1–S5. Quantities of marker compounds in Ridayarishta formulation have been mentioned in Table 1.

Table 2
Experimental parameters involved in fluorometric high-throughput screening assays.

CYP450 Isozymes	Positive inhibitors	Fluorogenic substrate	Fluorescent product	Incubation	Excitation	Emission
CYP1A2	Furafylline	CEC	CHC	15 min	410 nm	460 nm
CYP2C19	Tranylcypromine	 3-Cyano-7-Ethoxycoumarin	 3-cyano-7-hydroxycoumarin	30 min	410 nm	460 nm
CYP2D6	Quinidine	AMMC	AHMC	30 min	390 nm	460 nm
		 3-[2-(N,N-diethyl-N-methylamino)ethyl]- 7-methoxy-4-methylcoumarine	 3-[2-(N,N- diethylamino) ethyl]-7-hydroxy-4-methylcoumarine hydrochloride			
CYP3A4	Ketoconazole	BFC	HFC	30 min	409 nm	530 nm
		 7-benzyloxy-trifluoromethylcoumarin	 7-hydroxy-trifluoromethylcoumarin			

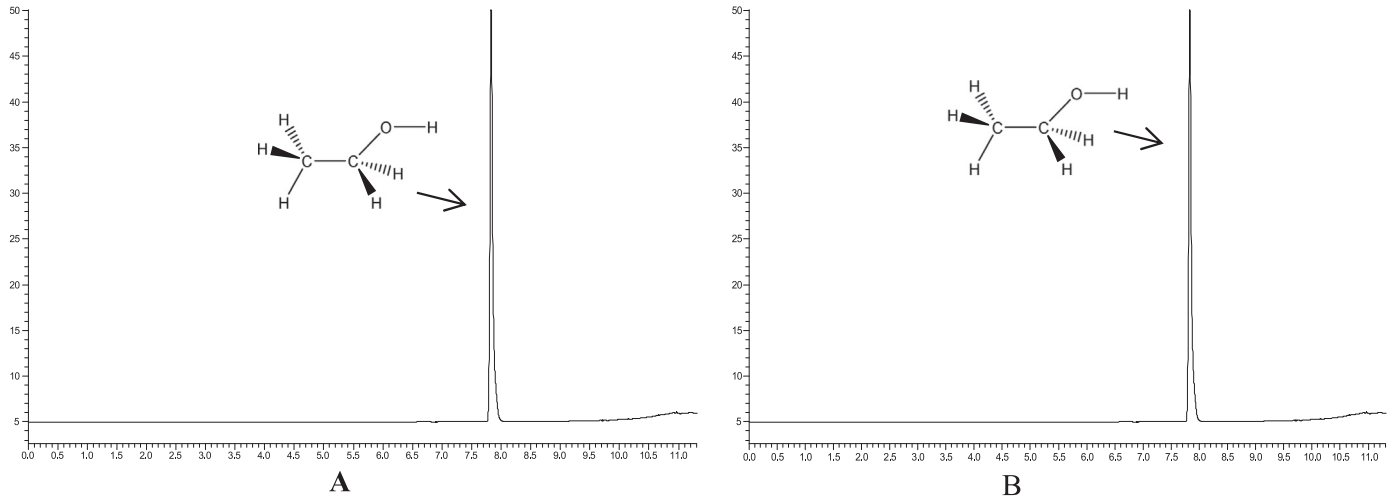


Fig. 1. Gas chromatogram of (A) standard ethanol and (B) Ridayarishta formulation.

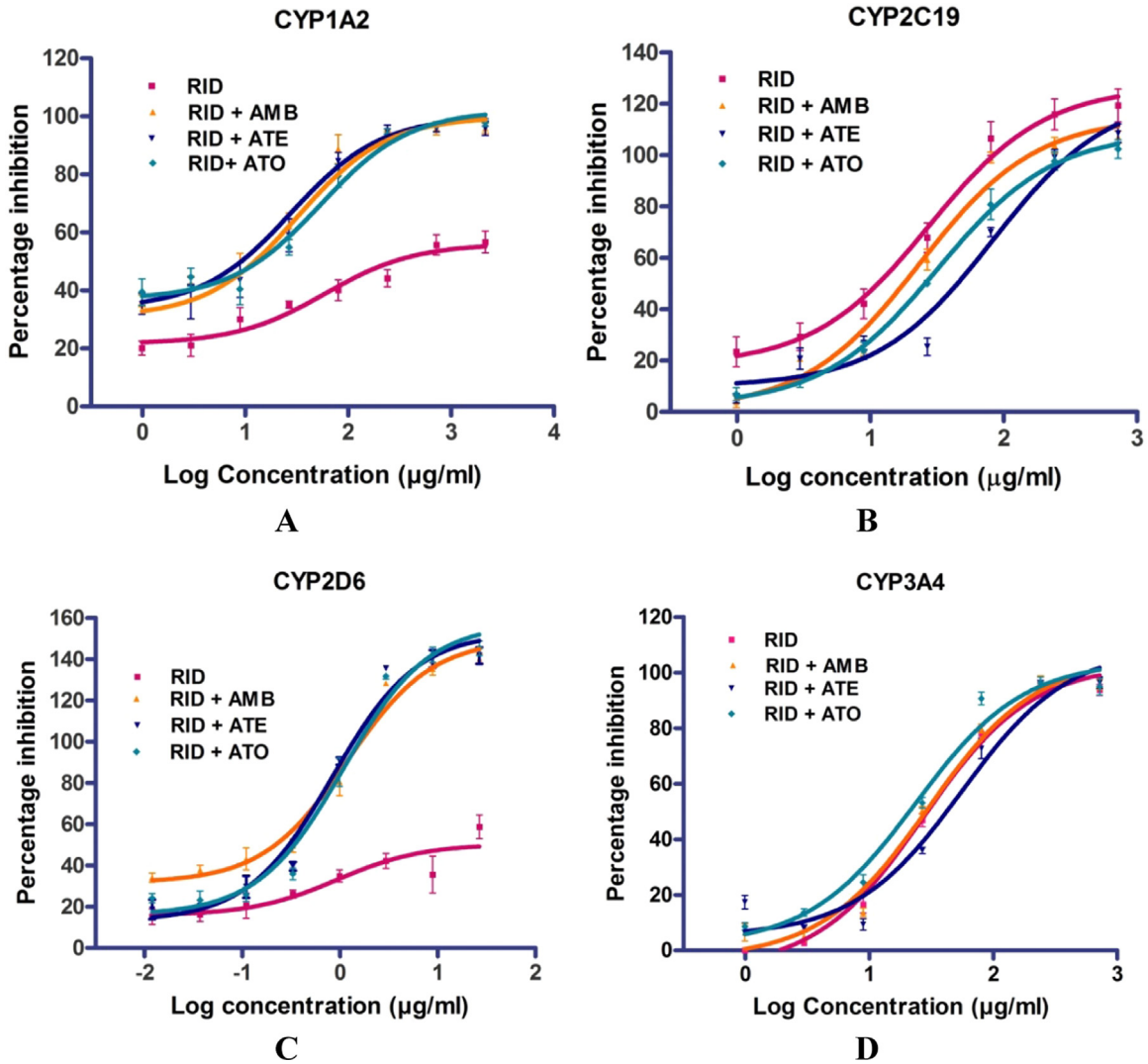


Fig. 2. Fluorometric high-throughput screening. Percentage inhibitory effects of Ridayarishta formulation (RID) alone and cocktail with amlodipine besilate (AMB), atenolol (ATE), and atorvastatin (ATO) on drug metabolizing isozymes (A) CYP1A2 (B) CYP2C19 (C) CYP2D6 (D) CYP3A4. [(Values are Mean ± SEM; n=3); RID+AMB=Cocktail of Ridayarishta formulation and amlodipine besilate; RID+ATE=Cocktail of Ridayarishta formulation and atenolol; RID+ATO=Cocktail of Ridayarishta formulation and atorvastatin].

3.2. Standardization of Ridayarishta formulation using GC

Ridayarishta formulation contains self-generated alcohol. Standardization of this Arista formulation with respect to ethanol content and to ensure absence of methanol is of prime importance to maintain proper quality of the product. Ethanol content of this Ayurvedic Arishta formulation using time saving, highly selective and specific GC analysis should be included in product specification by herbal drug manufacturer. Gas liquid chromatograms of ethanol and Ridayarishta formulation have been represented in Fig. 1. Presence of ethanol in Ridayarishta was confirmed by comparing retention time. Retention time was found to be 7.84 min. Linear calibration curve was found within range of 5–15% (V/V) Quantity of ethanol in the formulation was found to be 7.95 ± 0.023% (V/V).

3.3. Fluorogenic high-throughput screening (HTS) assays

Patients sometimes consumed herbal preparations along with conventional drugs for combination effect without knowledge of physician. It is of prime importance to identify and elucidate HDI potential between Ridayarishta formulation and conventional drugs. HTS assay on CYP450 activity using fluorescence technology based on fluorescent product formation is widely used in drug discovery process. Mammalian CYP450 isoforms are used to screen the activity of test drugs based on highly sensitive fluorescence inhibition techniques (Smith and Wilson, 2010).

3.3.1. Interaction study of Ridayarishta formulation with selected cardio protective and antihypertensive drugs

CYP450 metabolism mediated interaction of Ridayarishta formulation was studied with three commonly used antihypertensive drugs amlodipine besilate, atenolol and atorvastatin. Amlodipine besilate, a dihydropyridine calcium channel blocker prescribed for the treatment of ischemic heart disease and hypertension. It is metabolized through CYP3A family in liver (Zhu et al., 2014). Atenolol, a beta blocker actively restricts certain nerve impulses, thereby controlling the rate and force of contraction, consequently reduces blood pressure (de Abreu et al., 2003). Atorvastatin widely used in treatment of hypertension in general medicine. It is 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor and mainly metabolized through cytochrome CYP3A4 (Liu et al., 2010).

Inhibition potential of Ridayarishta formulation alone and amlodipine besilate, atenolol, atorvastatin cocktail against CYP1A2, 2C19, 2D6 and 3A4 isozymes have been represented in Fig. 2. Concentration of test drugs showed dose dependent inhibitory activity against all CYP450 isozymes. IC_{50} values of Ridayarishta and cocktail drugs have been shown in Table 3. Data indicated that the IC_{50} values of Ridayarishta formulation towards CYP1A2, CYP2C19, CYP2D6 and CYP3A4 were significantly higher as compared to their respective positive controls. There were not much deviation among IC_{50} values between Ridayarishta and drug cocktail. Further, cocktail of Ridayarishta formulation and selected antihypertensive and cardio protective drugs showed higher IC_{50} value than positive inhibitors. Results revealed that HDI potential of Ridayarishta and cocktail drugs were significantly less compared to respective positive inhibitors against CYP1A2, 2C19 and 3A4. In case of CYP2D6 negligible HDI was observed.

3.3.2. Interaction study of Ridayarishta formulation with selected anti-diabetic drugs

HDI potential of Ridayarishta formulation was assessed with anti-diabetic drugs metformin, glipizide and glimepiride. Metformin is not metabolized in liver and is excreted as such through urine. It disrupts the co-activation of nuclear pregnane X receptor (PXR), resulting in the down regulation of CYP3A4 gene expression (Krausova et al., 2011). Glipizide is a second-generation sulfonylurea indicated for the treatment of type 2 diabetes mellitus (Kobylińska et al., 2000). Glimepiride is metabolized in the liver by CYP2C family to an active hydroxy metabolite (Badian et al., 1996).

IC_{50} values of Ridayarishta formulation and drugs cocktail against CYP1A2, 2C19 2D6 and 3A4 have been represented in Table 3. Results revealed that Ridayarista and combination of anti-diabetic drugs showed higher IC_{50} value compared to positive inhibitors against all the tested CYP450 isozymes. Dose dependent percentage inhibitions of Ridayarishta and cocktail drugs on CYP isozymes have been depicted in Fig. 3. Test drugs showed sigmoidal dose response curve against CYP1A2, 2C19, 2D6 and 3A4. Hence, Ridayarishta formulation and cocktail drugs showed little inhibitory activity on CYP450; the HDI between Ridayarishta formulation and commonly used oral hypoglycemic drugs was negligible (Pandit et al., 2012; Ahmmed et al., 2016). Study confirmed that consumption of Ridayarishta along with selective hypoglycemic drugs is safe to consume with insignificant or negligible ADR.

Ridayarishta formulation showed significantly lower CYP1A2, 2C19, 2D6 and 3A4 inhibitory activity compared with respective positive inhibitors. Drugs cocktail showed significantly higher IC_{50} values than positive inhibitors against CYP1A2, 2C19 and 3A4. However, negligible CYP2D6 inhibitory activity was observed in cocktail drugs compared to quinidine. Concentrations of amlodipine besilate, atenolol, atorvastatin, metformin, glipizide and glimepiride were selected based on C_{max} values mentioned in literature, to prepare cocktail drug blend (Liu et al., 2009; de Abreu et al., 2003; Liu et al., 2010; Zhi-yu et al., 2012, Kobylińska et al.,

Table 3
IC_{50} value (μg/mL) of positive inhibitors, Ridayarishta formulation alone and cocktail with amlodipine besilate, atenolol, atorvastatin, metformin, glipizide, glimepiride on metabolism medicated by CYP1A2, 2C19 2D6, and 3A4.

Test samples	IC_{50}value (μg/mL)			
CYP isozymes	**CYP1A2**	**CYP2C19**	**CYP2D6**	**CYP3A4**
Positive inhibitors	0.63 ± 0.29	0.20 ± 0.05	0.002 ± 0.001	0.03 ± 0.02
Ridayarishta	13.80 ± 1.96*	14.34 ± 2.28*	0.90 ± 0.28*	32.06 ± 2.51*
Ridayarishta + Amlodipine besilate	12.74 ± 1.22*	22.03 ± 0.96*	0.33 ± 0.13ns	26.50 ± 1.01*
Ridayarishta + Atenolol	16.32 ± 1.52*	56.16 ± 1.44*	0.41 ± 0.03ns	47.12 ± 2.01*
Ridayarishta + Atorvastatin	20.64 ± 1.15*	26.67 ± 1.64*	0.43 ± 0.15ns	20.58 ± 1.99*
Ridayarishta + Metformin	12.94 ± 1.75*	15.66 ± 1.02*	0.63 ± 0.39**	13.07 ± 2.10*
Ridayarishta + Glipizide	17.98 ± 1.29*	40.79 ± 1.53*	0.43 ± 0.21ns	23.15 ± 1.62*
Ridayarishta + Glimepiride	13.87 ± 1.80*	30.88 ± 1.38*	0.45 ± 0.11ns	19.02 ± 2.39*

Values are represent as Mean ± SEM (n = 3). One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.
ns = non significance *versus* positive inhibitors.
* $P < 0.001$.
** $P < 0.01$.

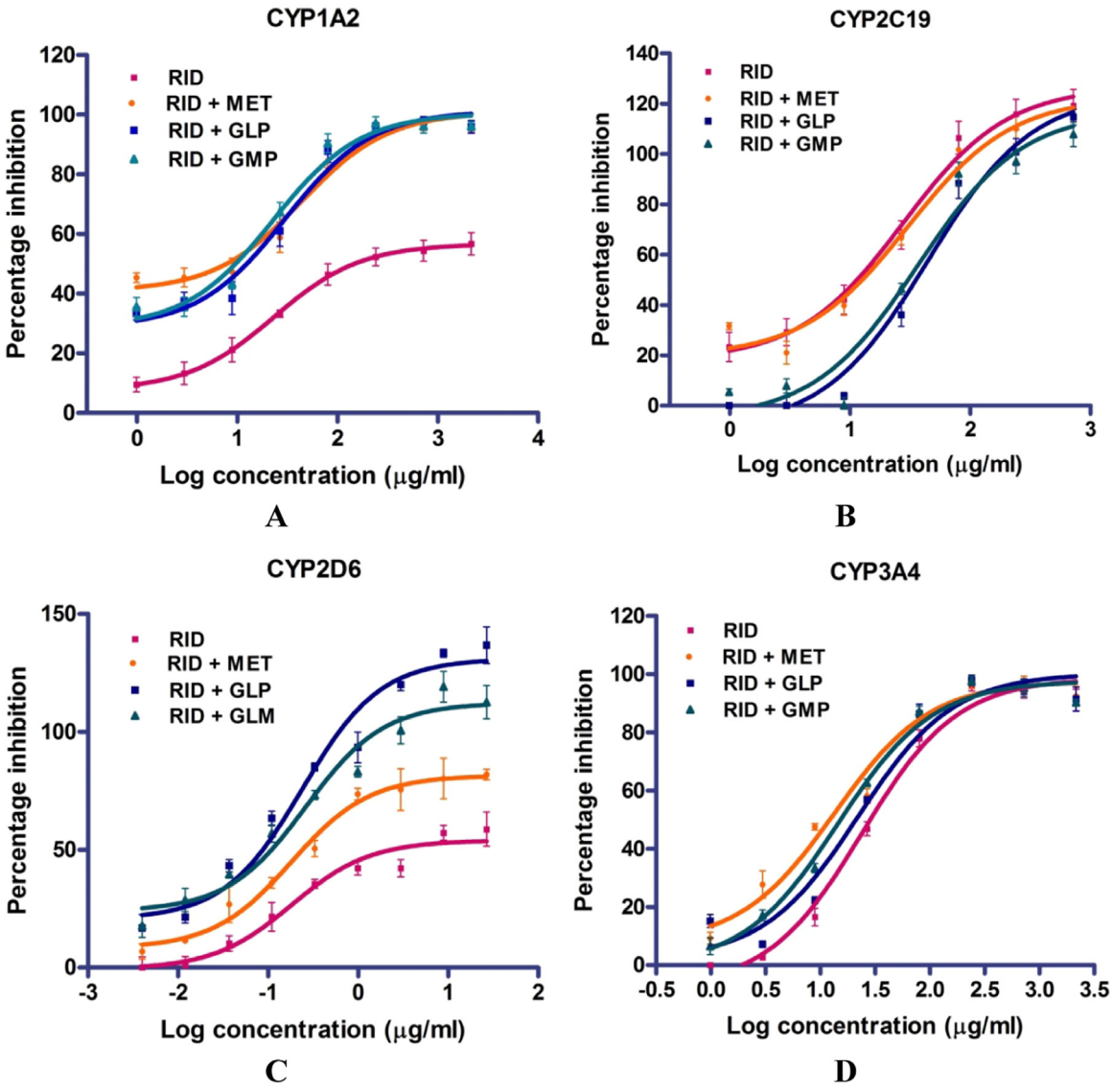


Fig. 3. Fluorometric high-throughput screening. Percentage inhibitory effects of Ridayarishta formulation (RID) alone and cocktail with metformin (MET) glipizide (GLP) glimepiride (GLM) on drug metabolizing isozymes (A) CYP1A2 (B) CYP2C19 (C) CYP2D6 (D) CYP3A4 [(Values are Mean ± SEM; n=3); RID+MET=Cocktail of Ridayarishta formulation and metformin; RID+GLP=Cocktail of Ridayarishta formulation and glipizide; RID+GLM=Cocktail of Ridayarishta formulation and glimepiride].

2000, Jovanović et al., 2006). It was predicted that with these concentrations, test samples have maximum CYP450 inhibitory activity. In case of CYP1A2 and 2C19, IC_{50} values were higher or near about same in cocktail drugs compared to Ridayarishta formulation. Further, insignificant human liver CYP3A4, 2D6 and 2C9 inhibitory activity of arjunic acid, arjunetin and arjun-genin, from *T. arjuna* have been proved by Varghese et al. (2015).

Results of the present study showed negligible or insignificant HDI of Ridayarishta formulation and cocktail with other selected conventional medicines. Our study established that Ridayarishta formulation is safe to consume without any potential HDI and may be recommended for the patients under cardio protective, antihypertensive and anti-diabetic medication. Further, detail metabolic pathway of Ridayarishta formulation, *in vivo* pharmacokinetic interaction, undesirable interactions with proteins or enzyme and mechanism based enzyme inhibition need to be addressed for better understanding of HDI.

4. Conclusion

CYP450 (CYP1A2, 2C19, 2D6 and 3A4) metabolism mediated HDI of Ridayarishta formulation and cocktail with commonly prescribed selective antihypertensive, cholesterol lowering and anti-diabetic drugs was negligible. It may be concluded that consumption of Ridayarishta formulation along with other medications can be regarded as safe. However, the detail mechanism based understanding of Ridayarishta metabolism and pharmacokinetic interaction study needs to be addressed further.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2016.07.061.

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