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Research Article

Standardization of Optimized Anti-diabetic Polyherbal Formulation by HPLC

Arun Kumar^{1*}, Ajudhia Nath Kalia², Harsimran Singh³¹Department of RIC, IKG Punjab Technical University, Kapurthala-144601, Punjab, India²Department of Pharmacognosy, Sri Sai College of Pharmacy, Pathankot-145001, Punjab, India³Department of Pharmacology, Sri Sai College of Pharmacy, Pathankot-145001, Punjab, India

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ABSTRACT

Herbal drugs are widely used to treat and prevent various diseases with more than 80% of people worldwide relying on them for their primary healthcare. Polyherbal formulations express high effectiveness in various diseases due to their synergistic effect. Herbal formulations are required to standardize in order to assess their quality, purity, and biological efficacy. The present study aimed to prepare a polyherbal formulation comprising of lyophilized ethanolic extracts of three well known anti-diabetic herbal drugs (*Momordica charantia*, *Andrographis paniculata* and *Withania somnifera*), and three formulations were developed in combination ratios (1:1:1, 2:1:2, and 2:2:1) respectively and to develop high-performance liquid chromatography (HPLC) method for assessment of purity, standardization and estimation of markers. In addition, developed formulations were optimized on the basis of oral glucose tolerance test (OGTT). Standardization of developed polyherbal formulations was performed by using respective biological markers (Vicine, andrographolide, withaferin-A and withanolide-A) by HPLC technique. HPLC was performed using RP-18, Merck column to study the presence and quantification of respective markers at a wavelength of 237 nm. The HPLC study revealed the presence of vicine, andrographolide, withaferin-A, and withanolide-A in quantifiable amounts in respective extracts. OGTT study was performed using normal albino Wistar rats at a drug dose of 500 mg/kg body weight. Rats were divided into six groups, including three combination ratios and standard (Metformin) group. An optimized ratio 2:2:1 showed a significant decrease in blood glucose levels compared with standard control rats.

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic disorders characterized by hyperglycemia due to impaired insulin secretion and variable degrees of peripheral insulin resistance. Symptoms of marked hyperglycemia include polydipsia, polyphagia, polyuria and weight loss.^[1] Chronic effects of hyperglycemia are categorized into macrovascular and microvascular complications.^[2] Long term diabetes may result in macrovascular complications like peripheral arterial disease, coronary artery disease, stroke and microvascular complications like neuropathy, retinopathy, nephropathy.^[3] According to World Health Organization (WHO), around 80% of the world population,

especially in developing countries, immensely uses traditional and alternative medicines as a source of their primary health care.^[4,5] The anti-diabetic effects of several plant extracts, herbal formulations, polyherbal formulation and their bioactive components have been identified and characterized.^[6] In the case of a single drug dose, the chances of toxicity are higher because the dose of the drug is increased to attain more therapeutic effects, which subsequently increases the concentration of unidentifiable and unwanted phytoconstituents. Moreover, a single drug generally exerts its therapeutic effect by one or two mechanisms. On the other hand, polyherbal therapies are much safer comparatively because of low-

*Corresponding Author: Arun Kumar

Address: Research Scholar, Department of RIC, IKG Punjab Technical University, Kapurthala-144601, India

Email ✉: arunbehl85@gmail.com

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dose combinations of different drugs and much lower amounts of the other phytoconstituents. Additionally, polyherbal therapies have multifarious actions due to different drugs that may synergistically provide adequate therapeutic relief in a diseased state.^[7,8] Herbal formulations are required to be standardized to assess their quality and efficacy based on concentration of their therapeutic active constituents^[9] and standardization is important as a means to justify their acceptability in the modern system of medicine.^[10] This could be attained by developing a reliable protocol for evaluating and analyzing herbal products by using modern scientific analytical tools.^[11] WHO emphasizes the qualitative and quantitative methods for characterizing the samples and quantifying the biomarkers and/or chemical markers.^[12]

M. charantia (Karela) is a member of the cucumber family (Cucurbitaceae), also known as bitter melon.^[13] The fruit of *M. charantia* was reported to have anti-diabetic potential^[14] antihyperlipidemic, anticarcinogenic,^[15] anti-inflammatory, hepatoprotective, anti-viral, antipyretic, and antimalarial activities.^[16] Vicine, a glycol-alkaloid (Fig. 1a) present in the fruit used as a biological marker for Karela products, which acts on insulin secretion and glycogen synthesis.^[17] *A. paniculata* (Kalmegh) belongs to the family Acanthaceae, commonly known as King of Bitters, is an annual, branched, erect herb running half to one meter in height.^[18] It has shown to possess wide spectrum of pharmacological activities like antihyperglycemic, antihyperlipidemic,^[19] hepatoprotective,^[20] cytotoxicity,^[21] anti-inflammatory,^[22] immunomodulatory and anti-viral.^[23] Andrographolide (Fig. 1b) is a major bioactive marker found in all parts of the Kalmegh plant, reported to possess hypoglycemic effect in normal and STZ-diabetic rats^[24] and reported to improve the absorption of glucose in isolated soleus muscle of STZ-diabetic rats by increasing the mRNA and protein levels of GLUT4.^[25] *W. somnifera* (Ashwagandha) is an erect, evergreen, branched shrub from the Solanaceae family.^[26] Numerous studies revealed that roots of *W. somnifera* had been shown to exhibit various pharmacological activities like anticancer,^[27] anti-microbial,^[28] hepatoprotective,^[29] anti-diabetic,^[30] antioxidant,^[31] anti-inflammatory,^[32]

cardioprotective,^[33] anti-arthritis,^[34] neuroprotective activity^[35] and immunomodulatory potential.^[36] Withaferin-A and withanolide-A, steroidal lactones (Fig. 1c and d), are the important withanolides present in the roots of Ashwaganda reported to overcome the multiple low doses possess (MLD)-STZ induced inflammatory insult and even prevented diabetogenesis^[37] and possess immunomodulatory potential.^[38]

The OGTT reflects the ability of pancreatic β -cells to secrete insulin and the sensitivity of tissues to insulin. Moreover, the OGTT has often been used to evaluate β -cells function and insulin resistance.^[39] In the present study, lyophilized extracts of *M. charantia*, *A. paniculata* and *W. somnifera* were combined in different ratios, and an optimized ratio was selected based on preliminary screening by OGTT. Lyophilization of extracts was done in order to improve the physical state, bioavailability, and stability of the thermolabile constituents.^[40] Further HPLC method was developed for simultaneous determination of vicine, andrographolide, withaferin-A, and withanolide-A in different combination ratios.

MATERIALS AND METHODS

Drug, Chemicals, and Instruments

The dried fruit of *M. charantia*, whole plant of *A. paniculata* and roots of *W. somnifera* were procured from crude drug supplier Herb Heal Consortium Pvt. Ltd., Ramtirath road, Amritsar, Punjab, and crude drugs were authenticated by Dr. Bikarma Singh, Herbarium & Crude Drug Repository Division, CSIR-IIIM, Canal Road, Jammu (Ref. no.:CSIR-IIIM/JAH/2020/07). Vicine was procured from Natural Remedies Pvt. Ltd., Bangalore. Andrographolide, withaferine-A and withanolide-A were procured from CSIR-IIIM, Canal Road, Jammu. HPLC (WATERS, W2996), rotary evaporator (Buchi Rotavapor R-210, Switzerland), lyophilizer were used during the study. Solvents used in HPLC system were of HPLC grade, and other reagents used were of analytical grade.

Extraction and Lyophilization

The selected plant materials were shade dried, coarsely powdered, and stored in an airtight container. Each drug was extracted with ethanol (80% v/v) by soxhlation method. The prepared extracts were concentrated using a rotary evaporator at 45°C under vacuum. The extracts were freeze-dried at -20°C for 12 hours then lyophilized using a lyophilizer. The lyophilized extracts were stored in an airtight container and kept in the desiccators for further use.

Development and Optimization of Polyherbal Formulation (PHF)

Polyherbal formulations were developed by combining the lyophilized extracts of selected drugs in different

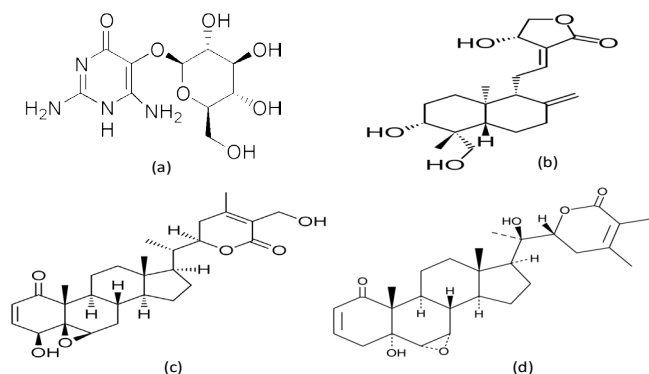


Fig. 1: Chemical structures of Vicine (a), Andrographolide (b), Withaferin-A (c) Withanolide-A (d)

experimental ratios (Table 1) and were optimized on the bases of OGTT study in normal rats.

Experimental Animals

Albino Wistar rats weighing 180–240 g were housed at Central Animal House, Panjab University, Chandigarh. The rats were housed in clean, sterile, polypropylene cages under standard laboratory conditions ($25 \pm 2^\circ\text{C}$, 60–70% humidity) and 12 hours light/dark cycle with standard chow (Aashirwad Industries, Mohali, India) and water provided *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Panjab University (PU/45/99/CPCSEA/IAEC/2020/424). The experiment was conducted according to CPCSEA guidelines for the use and care of experimental animals.

Oral Glucose Tolerance Test (OGTT) Study

Overnight fasted rats were used to conduct OGTT study. Hyperglycemia was induced by the administration of glucose (2g/kg) to normal rats. The rats were divided into six groups each containing six animals. Group I Normal control was administered with 0.5% w/v carboxymethylcellulose solution, Group II Positive control was administered with glucose (2g/kg) and Group III-VI were received PHF A, PHF B, PHF C, and standard (Metformin) respectively at single dose level (500 mg/kg per oral) prior to one hour of the glucose load. The blood was obtained by pricking the tail vein at times -60, 0, 60, and 120 minutes after glucose administration. The serum glucose level (SGL) was estimated within 30 minutes of withdrawal of the blood sample by the glucose oxidase-peroxidase method.^[41]

Acute Toxicity Study

Acute oral toxicity study was conducted as per revised organization for economic co-operation and development

Table 1: Different Combination ratios of Lyophilized extracts of *Momordica charantia*, *A. paniculata*, and *W. somnifera*

S.No.	Formulation	<i>Momordica charantia</i> (Karela)	<i>Andrographis paniculata</i> (Kalmegh)	<i>Withania somnifera</i> (Ashwaganda)
1.	PHF A	1	1	1
2.	PHF B	2	1	2
3.	PHF C	2	2	1

Table 2: Effect of different formulations and metformin at the dose of 500 mg/kg body wt. on serum glucose level of glucose loaded normal rats (^a = $p < 0.05$ vs positive control)

Groups	Serum glucose levels (mg/dL)			
	-60 mins	0 min	60 min	120 min
Normal control	91.3 $\pm$ 12.48	91.5 $\pm$ 12.72	92.10 $\pm$ 13.92	91 $\pm$ 12.85
Positive control	90.8 $\pm$ 14.94	91.3 $\pm$ 12.30	142.80 $\pm$ 9.90 (55.04%)	108.5 $\pm$ 10.94
PHF A (500 mg/kg b.wt)	92.16 $\pm$ 12.49	90.0 $\pm$ 8.12	118.83 $\pm$ 10.87 (29.02%) ^a	98.5 $\pm$ 10.17
PHF B (500 mg/kg b.wt)	91.83 $\pm$ 11.05	88.33 $\pm$ 9.43	113.50 $\pm$ 11.32 (23.23%) ^a	96.66 $\pm$ 10.21
PHF C (500 mg/kg b.wt)	91.66 $\pm$ 10.80	86.33 $\pm$ 8.84	105.33 $\pm$ 11.77 (14.36%) ^a	90.33 $\pm$ 8.40
Metformin (500 mg/kg b.wt)	89.83 $\pm$ 11.26	89.66 $\pm$ 8.91	109.00 $\pm$ 10.00 (18.34%) ^a	94.66 $\pm$ 8.64

(OECD) guidelines on the rats with the OGTT optimized formulation. The overnight fasted rats were administered different doses up to 5 g/kg of the formulation. The rats were observed for 14 days for any kind of behavioral changes, locomotion, and mortality.^[42]

Sample Preparation

Different extracts and their combinations were studied for the presence of their respective markers using HPLC. Each extract (25 mg) was dissolved in 5 mL of methanol (HPLC grade), and the same ratio (5 mg/mL) was prepared for three combination ratios. All the samples were diluted suitably, followed by filtration through a 0.45 μm membrane filter. All the bioactive markers were dissolved as 1mg/mL in methanol (HPLC grade). The sample solutions were injected in triplicate for the analysis.

Instrumentation

The HPLC system has consisted of Water 2996 HPLC system provided a binary pump, an automatic sampler, degasser, a diode array detector, thermostatic column oven and the RP-18, Merck column (4×250 mm, 5 μm) with mobile phase, and comprised of a mixture of acetonitrile and water (1:1) running with a flow rate of 0.5 mL/min. Filtration of mobile phase was performed using 0.45 μm filter before use. The column's temperature was maintained at 30°C to dispense sharpness to the eluting peak. The chromatogram was recorded at 237 nm.

RESULTS

Yield of Different Extracts

The percentage yield of three different 80% ethanolic extracts were obtained as *M. charantia* (12.54% w/w), *A. paniculata* (8.48% w/w) and *W. somnifera* (10.45% w/w).

Optimization of Polyherbal Formulation using OGTT in Normal Rats

The normal vehicle-treated rats have shown non-significant changes in SGL. The result showed 55.04 and 18.34% rise in SGL after 60 minutes of glucose administration in the positive control and metformin pretreated group, respectively (Table 2). Whereas, group of animals pretreated with developed formulations (PHF A, PHF B, PHF C) at dose 500 mg/kg have shown a rise in SGL



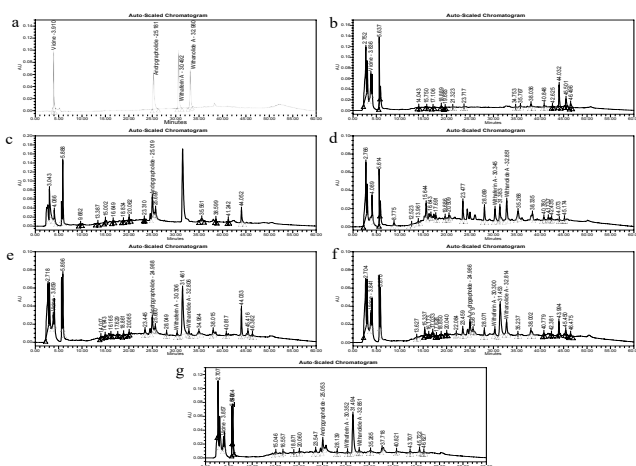


Fig. 2: HPLC chromatogram of (a) Standard mixture (b) *M. charantia* extract (c) *A. paniculata* extract (d) *W. somnifera* extract (e) Combination ratio (1:1:1) (f) combination ratio (2:1:2) (g) Combination ratio (2:2:1)

by 29.02, 23.23, and 14.36% respectively in comparison. The OGTT study results showed that PHF C has the most potent antihyperglycemic combination compared to other combination ratios evaluated even at par with the standard drug.

Acute Toxicity Study

The treatment of rats with polyherbal formulation has not shown any sign of behavioral changes up to the dose of 5 g/kg body wt.

Retention Time and Quantification of markers in different extract/combination

The retention time of vicine, andrographolide, withaferine-A and Withanolide-A in the standard mixture were noted to be 3.910, 25.181, 30.492 and 32.980 (minutes) respectively as shown in the chromatogram. These standard compounds showed a peak at a retention time of vicine (3.836), andrographolide (25.181), withaferine A (30.492), and withanolide A (32.980) in individual extracts (Fig. 2). The percentage of vicine in *M. charantia* extract was found to be 1.81%. The percentage of andrographolide in *A. paniculata* extract was found to be 0.35%. The percentage of withaferine A and withanolide A in *W. somnifera* extract was found to be 0.23% and 0.10%, respectively. In PHF A, standard compounds showed a peak at the retention time of vicine (3.859), andrographolide (24.988), withaferine A (30.306), and withanolide A (32.806) with an amount of vicine (0.50%), andrographolide (0.11%), withaferine A (0.02%), and withanolide A (0.03%). In PHF B, standard compounds showed a peak at a retention time of vicine (3.841), andrographolide (24.986), withaferine A (30.300), and withanolide A (32.814) with an amount of vicine (0.72%), andrographolide (0.09%), withaferine A (0.04%) and withanolide A (0.15%). In PHF C, standard compounds showed a peak at a retention time of vicine (3.857), andrographolide (25.053), withaferine A (30.352),

and withanolide A (32.851) with an amount of vicine (0.58%), and rographolide (0.12%), withaferin A (0.01%) and withanolide A (0.03%).

DISCUSSION

Polyherbal formulation exerts its therapeutic effect by a different mechanism of action due to the presence of multiple active constituents from different plants, thereby producing pleiotropic effects to prevent disease progression and, consequently, treat the disease condition.^[43] In the present study, polyherbal formulation containing ethanolic extracts of *M. charantia*, *A. paniculata* and *W. somnifera* were prepared in different ratios (1:1:1), (2:1:2) and (2:2:1) respectively. A study reported that ethanolic extracts of the *M. charantia* at the dose 200 mg/kg increased glucose uptake by inhibiting enzymes involved in the glycolysis pathways including glucose-6-phosphatase and fructose 1, 6-diphosphatase^[44] and stimulate glucose-6-phosphatase dehydrogenase in the liver.^[45] A study revealed that ethanolic extracts of *A. paniculata* showed glucose-lowering and hypolipidemic effects in high-fat-fructose fed rats at dose 400 mg/kg twice daily, and active compound andrographolide at dose 4.5 mg/kg twice daily showed a significant decrease in blood glucose and improved diabetic rat islet and beta cells.^[46] It has been demonstrated in a study that *W. somnifera* root extract at 200 mg/kg dose showed a hypoglycaemic effect in alloxan-induced diabetic rats.^[47] A study reported that administration of vicine lowered blood glucose level below normal in non-diabetic fasting rats.^[48] On the other hand, andrographolide has been reported to decrease the plasma glucose concentration when administered orally in a dose-dependent manner in streptozotocin (STZ)-induced diabetic rats where andrographolide (1.5 mg/kg) significantly attenuated the increased plasma glucose level in an intravenous glucose challenge test in normal rats.^[49] Withaferin-A and withanolide-A increased glucose uptake in dose dependant manner in rat myotubes (L6) and adipocytes (3T3-L1).^[50] Our investigation supports these contentions that all the three combination ratio of these different extracts showed antihyperglycemic potential. PHF C (500 mg/kg) has shown a 14.36% rise in SGL just at par with PHF A and PHF B, shown better results than standard drug, metformin (500 mg/kg) in OGTT study. However, all the groups of animals almost normalized serum glucose levels within 2 hours that indicate the pancreas' health and its capacity to clear out the glucose load from the body. The HPLC results confirmed that extracts of *M. charantia*, *A. paniculata*, and *W. somnifera* contained their active constituents in respective amounts.

CONCLUSION

In this study, three herbal drug extracts (*M. charantia*, *A. paniculata* and *W. somnifera*) and their polyherbal

formulations in the ratio of (1:1:1), (2:1:2) and (2:2:1) respectively were prepared. These formulations were further studied for the presence of their respective markers such as vicine, andrographolide, withaferin-A, and Withanolide-A by HPLC and were found to be present in a quantifiable amount in each of them. Polyherbal formulations were optimized using OGTT study in rats, which revealed that formulation in the ratio of (2:2:1) is the most promising in terms of lowering blood glucose levels. Therefore, it may be suggested that this anti-diabetic potential of polyherbal herbal formulation (2:2:1) could be attributed to active constituents such as vicine andrographolide withaferine-A, and withanolide-A. This study is first of its kind as these polyherbal formulations have never been prepared and studied until date, which could provide advantages like synergistic anti-diabetic potential and reduced need for drugs compared to administration of the individualized extract. Further studies are in progress to study the effect of optimized polyherbal formulation (2:2:1) *in vivo* and will elucidate the molecular mechanisms responsible for its anti-diabetic potential.

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