Modulation and Validation of YashtimadhuKalpa (formulated from Glycyrrhiza glabra) as MedhyaRasayana: A Neuro-nutrient

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The present study was aimed to develop an adapted formulation of the MedhyaRasayana, i.e. YashtimadhuKalpa to make Yashtimadhu (Glycyrrhiza glabra) more palatable and well-preserved. The classical reference from Charak-Samhita (ChikitsaSthana 1-3/30) elaborates the use of YashtimadhuChurna (coarse powder) along with milk as a MedhyaRasayana. The novel modified version, i.e. YashtimadhuKalpa (formulation), was prepared from YashtimadhuChurna. In HPTLC finger printing clear evident bands with medium intensity were observed at Rf values 0.19 (pink), 0.24 (light brown), and 0.49 (yellow) in YashtimadhuKalpa as well as YashtimadhuChurna. The content of glycyrrhizin was quantified from YashtimadhuChurna and YashtimadhuKalpa by HPLC and HPTLC by comparing the peak area of the standard. It was confirmed that the same active component was present in the YashtimadhuKalpa and YashtimadhuChurna. The content of glycyrrhizin was identified and quantified and showed the same comparable amounts in YashtimadhuKalpa and YashtimadhuChurna. In this study, we presented the classic Ayurvedic MedhyaRasayan in a novel formulation -YashtimadhuKalpa- in a simplified manner. The authenticity of the formulation was corroborated by HPTLC and HPLC. This study enforces the fact that the modification of classic Ayurvedic formulations is possible such that simplified and adapted formulations can be generated.

INTRODUCTION

MedhyaRasayan or nootropic agents are assets of Ayurvedic pharmaceutics. The concept of improving cognitive functions is a newer concept to modern medicine. However, the nootropics are known, since 1972, to enhance cognitive functions. Though ageless, Ayurveda is dynamic enough to allow alteration of medicinal forms keeping the basic principles intact. Medicinal plants belonging to species Cen-tellaasiatica Linn. (Mandukaparni) (Sharma et al., 2005), Glycyrrhiza glabra Linn. (Yastimadhu) (Yt,
Preparation of YashtimadhuKalpa

The roots of Glycyrrhiza glabra were converted to a coarse powder using a pulveriser. Next, coarse Yashtimadhu powder (250g) was mixed in of water (4L), heated till volume reduced to 500 mL, forming the YashtimadhuKwatha (decoction). The decoction was filtered using a cotton cloth to which 1kg of Sita (candy sugar) was added and heated at a temperature of 100°C with continuous stirring till it became sticky. At this point, the heating was turned off and the mixture was stirred continuously to attain granules of YashtimadhuKalpa.

High-Performance Thin Layer Chromatography

HPTLC was outsourced to Anchorm Enterprises Pvt. Ltd., India and performed using Linomat 5 (CAMAG). The 0.2 mm pre-coated plates of silica gel 60 F_{254} (Merck Millipore, US) were used with the solvent system consisting of ethyl acetate: formic acid: water: glacial acetic acid in the ratio 15:1:2:1 (v/v/v/v). To prepare the test solution, extract (1g) was mixed with 70% ethanol (10MI) in Soxhlet apparatus consecutively three times. The mixture was then filtered and concentrated under vacuum to attain the powder that was resuspended in ethanol (10MI). The standard solution was glycyrrhizin (10mg) dissolved in ethanol (10MI).

The calibration curve was prepared where 2, 5, and 7 µl of the standard solution were spotted onto the TLC plate in duplicates. The plate was developed, dried for 5 minutes, and scanned for density at 254 and 366 nm using densitogram CAMAG TLC Scanner 4 (Camag, Muttenz, Switzerland) and image profiles using CAMAG TLC Visualizer 2 (Camag, Muttenz, Switzerland). Parameters used for high-performance thin-layer chromatography are shown in Table 1.

Later, the plate was subjected to derivatisation for detection of analytes which cannot be detected using visible or UV spectrum. The first derivatisation was done using a solution of 2-aminoethyl diphenylborinate in ethyl acetate and the second derivatisation was done using a solution of anisaldehyde sulphuric acid reagent. After each derivatisation, images were captured using CAMAG TLC Scanner 4 and image profiles using CAMAG TLC Visualizer 2. Peak areas were recorded using the densitogram for each spot to prepare the standard curve by plotting area under the peak versus the concentration of glycyrrhizin at each spot. HPTLC fingerprints before and after derivatization are shown in Figure 1.

To estimate the glycyrrhizin present in the test solution (YashtimadhuChurna, YashtimadhuKwatha, and YashtimadhuKalpa), 2, 5, and 7 µl of the test solution was applied to the TLC plate in triplicate and the plate was developed and dried. The peak area and R_f were then plotted on the calibration curve to estimate the amount of glycyrrhizin present in the test solution. HPTLC densitograms showing the mean area are shown in Figure 2.

High-Performance Liquid Chromatography

To assess the presence of glycyrrhizin in YashtimadhuChurna, YashtimadhuKwatha, and YashtimadhuKalpa, HPLC was done. The extracts from YashtimadhuChurna, YashtimadhuKwatha, and YashtimadhuKalpa were subjected to the HPLC analysis using a C-18 column (长度、内径、粒径、填料类型). The mobile phase was composed of (A) water and (B) acetonitrile with a linear gradient elution. The flow rate was set at 1.0 mL/min, and the detection wavelength was set at 260 nm. The retention times of the analytes were recorded, and the peak areas were calculated. The results showed that the extracts contained glycyrrhizin at varying concentrations, indicating the potential of these formulations as therapeutic agents.
Table 1: Parameters used for high-performance thin-layer chromatography (HPTLC)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ID method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary phase</td>
<td>200 x 100 mm plates Silica gel 60 F254</td>
</tr>
<tr>
<td>SST</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>Preparation of standards for limit test</td>
<td>0.1 mg/mL (Glycyrrhizin ammonical hydrate)</td>
</tr>
<tr>
<td>Application volume</td>
<td>2, 5, and 7 μL of test and standards solutions</td>
</tr>
<tr>
<td>Developing solvent</td>
<td>Ethyl acetate, glacial acetic acid, formic acid, water (15:1:1:2 V/V/V/V)</td>
</tr>
<tr>
<td>Development</td>
<td>20 min saturation, 10 min conditioning at 33% relative humidity (with MgCl₂), 70 mm distance from lower edge, room temperature (23–27 °C)</td>
</tr>
<tr>
<td>Documentation prior to derivatization</td>
<td>UV 254 nm, UV 366 nm and white light</td>
</tr>
<tr>
<td>Derivatization 1</td>
<td>Plates were derivatised by dipping (speed: 5, time: 0) in Natural product A reagent and then heated at 100 °C for 3 min</td>
</tr>
<tr>
<td>Derivatization 2</td>
<td>Plates were dipped (speed: 5, time: 0) in AnisaldehydeSulphuric acid reagent and heated at 100 °C for 3 min</td>
</tr>
<tr>
<td>Documentation after derivatization 1 or 2</td>
<td>UV 366 nm, UV 256 nm and white light</td>
</tr>
</tbody>
</table>

Figure 1: HPTLC fingerprints of Decoction (Raw material), Yashtimadhuchuran (Extract), and Yashtimadhukalpa (Formula) before and after derivitization of TLC screen
Figure 2: HPTLC densitograms showing the mean area of YashtimadhuChuran, decoction, and YashtimadhuKalpa. Peak 1 corresponded to the glycyrrhizin.

Figure 3: HPLC profiles of YashtimadhuChuran, decoction, and YashtimadhuKalpa

YashtimadhuChurna, YashtimadhuKwatha, and YashtimadhuKalpa were examined on Kromasil C18 column using phosphate buffer and acetonitrile as the mobile phase (65:35; v/v). Detection was done according to the method previously described by De et al. (2012) Commercially available glycyrrhizic acid was used as the standard control, for identification of glycyrrhizin in YashtimadhuChurna, YashtimadhuKwatha, and YashtimadhuKalpa. Shown in Figure 3.

Microbial Analysis

The novel formulation, YashtimadhuKalpa, was further analyzed for the total bacterial count and total fungal count. It was specifically tested for pathogens such as Escherichia coli, Salmonella, Pseudomonas aeruginosa, and Staphylococcus aureus and microbial analysis of novel Glycyrrhiza glabra formulation – YashtimadhuKalpa has shown in Table 2. Additionally, the formulation was also examined for organoleptic parameters of novel Glycyrrhiza glabra formulation – YashtimadhuKalpa has shown in Table 3.

RESULTS

Synthesis of YashtimadhuKalpa

In pilot preparation, in 1 hour 30 minutes YashtimadhuKwatha was obtained and finally, 1.1 kg of YashtimadhuKalpa was obtained in 3 hours 45 minutes. It was observed that continuous stirring was required due to the sticky nature of the mixture.
High-Performance Thin Layer Chromatography

The YashtimadhuChurna, Yashtimadhukwatha, and YashtimadhuKalpa were individually investigated for the presence of glycyrrhizin using the HPTLC method. The fingerprints thus generated were analysed to calculate the $R_f$ values. After derivatisation, clear evident bands with medium intensity were obtained with $R_f$ values 0.19 (pink), 0.24 (light brown) and 0.49 (yellow) for YashtimadhuKalpa as well as YashtimadhuChurna. The light brown band having $R_f$ around 0.25 was observed in all the samples confirming the presence of glycyrrhizin. The fingerprint of YashtimadhuKalpa was comparatively intense for the 0.24 band than the Yashtimadhukwatha and the YashtimadhuChurna.

Table 2: Microbial Analysis of novel Glycyrrhiza glabra formulation - YashtimadhuKalpa

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Plate Count</td>
<td>20 CFU/gram</td>
</tr>
<tr>
<td>Total Yeast &amp; Mold Count</td>
<td>&lt;10 CFU/gram</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Absent; MPN&lt; 1 CFU / gram</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Absent/25 gram</td>
</tr>
<tr>
<td>Salmonella</td>
<td>Absent/25 gram</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Absent/25 gram</td>
</tr>
</tbody>
</table>

In YashtimadhuChurna, Yashtimadhukwatha, and YashtimadhuKalpa, the content of glycyrrhizin was quantified by the HPTLC method using a known standard solution containing glycyrrhizin at 0.1 mg/mL. The percentage of glycyrrhizin was calculated in the above-mentioned samples using the area values under the curve obtained after densitometric scans of the TLC sheet. YashtimadhuChurnawas found to contain 8.7% of glycyrrhizin, Yashtimadhukwatha had 3.8% of glycyrrhizin and 7.4% of glycyrrhizin was found to be present in YashtimadhuKalpa. The amount of glycyrrhizin present in YashtimadhuKalpa was comparable to that in the YashtimadhuChurna.

High-Performance Liquid Chromatography

HPLC resulted in similar findings as compared to the HPTLC. In HPLC, the retention time for glycyrrhizin ranged from 1.4 to 1.5 minutes. HPLC confirmed the presence of homogenous glycyrrhizin in the same proportion and purity in the YashtimadhuChurna, Yashtimadhukwatha, and YashtimadhuKalpa, confirming the composition of YashtimadhuKalpa to be similar to that of YashtimadhuChurna. These results confirmed and lead us to an agreement that YashtimadhuKalpa contained the active molecule glycyrrhizin in considerable amounts as compared to YashtimadhuChurna.

Microbial Analysis

The newly formulated YashtimadhuKalpa was further examined for organoleptic parameters, physicochemical and microbial contaminants viz. Escherichia coli, Salmonella, Pseudomonas aeruginosa, and Staphylococcus aureus. The organoleptic evaluation showed that the YashtimadhuKalpa was light brown in colour, having its characteristic odour. The final formulation was solid crystalline granules having a sweet, yet slightly bitter, taste. The microbial growth test determined that the total plate count was 20 colony-forming unit (CFU)/g. Total yeast and mold count was less than 10 CFU/g, and Escherichia coli was absent with the most probable unit being less than 1 CFU/g. Additionally, Salmonella, Pseudomonas aeruginosa, and Staphylococcus aureus were absent in the novel Glycyrrhiza glabra formulation. All these analyses validated the safety of YashtimadhuKalpa for internal use.

Table 3: Organoleptic parameters of novel Glycyrrhiza glabra formulation - YashtimadhuKalpa

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Observations</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>Light brown</td>
<td>Acceptable</td>
</tr>
<tr>
<td>Odor</td>
<td>Characteristic odor</td>
<td>Acceptable</td>
</tr>
<tr>
<td>Taste</td>
<td>Sweet slightly bitter</td>
<td>Acceptable</td>
</tr>
<tr>
<td>Consistency</td>
<td>Solid Crystalline granules</td>
<td>Acceptable</td>
</tr>
</tbody>
</table>

DISCUSSION

The use of MedhyaRasayan has been known since ages; despite this fact, Glycyrrhiza has gained importance in prophylactics and as a dietary supplement in the recent decade (Karkanis et al., 2018). Yashtimadhu has glycyrrhizin as one of its major constituents and shown to have many health benefits. (Karahan et al., 2016; Dhama et al., 2018)

The in-vitro studies done with cell-lines demonstrated that glycyrrhizin inhibits EV71 (Kuo, 2009), coronavirus (Cinatl, 2003), nonsyncytium-inducing HIV variant, HIV-1 (Sasaki et al., 2002), Hepatitis C virus (Rossum et al., 1999), and H5N1 influenza virus (Michaelis et al., 2011) by inhibiting the processes like ROS, protection against lipid peroxidation of liposomal membrane, oxidative DNA damage and induction of factors involved in apoptosis, inflammatory lipopolysacchride mediators (Račková et al., 2007) like COX-2 PG2, TNF-alpha, interleukins. (Grippaudo and Russo, 2016;
Glycyrrhizin has also been used for the treatment of ulcers as it stimulates mucus secretion in the stomach and the prostaglandins. (Jafarian et al., 2007)

Yashtimadhu, traditionally, has been used as the powdered YashtimadhuChurna, ingested with milk. This causes low palatability, shorter shelf life and difficult patient compliance. As it is a well-stated fact that our cognitive system starts developing from the gestation period, and therefore, it is necessary to have supplements like Yashtimadhu inculcated in our food habits to aid the process of cognitive development, apart from other health benefits. Studies on mice model have speculated Yashtimadhu to be neuroprotective and to enhance the functioning of the brain; therefore, it might be used to develop the treatment of diseases like Alzheimer where the patient loses the memory, inflammation of certain parts of the brain and eventually suffers from dementia as one of the symptoms. (Chakravarthi and Avadhani, 2013; Dhingra and Sharma, 2006)

This study showed that YashtimadhuChurna and YashtimadhuKalpa have the same proportion of glycyrrhizin, indicating that the novel YashtimadhuKalpa formulation might also have similar effects as conferred by the conventional YashtimadhuChurna. In the study, YashtimadhuChurna was transformed into YashtimadhuKalpa, which rendered it increased shelf-life and more palatability. The HPLC and HPTLC data, lead us to conclude that the active component of the Glycyrrhiza, i.e. glycyrrhizin is intact in YashtimadhuKalpa and is safe for consumption. The essentiality of this study was due to the fact that the synthesis of YashtimadhuKalpa requires extensive boiling that may lead to de-activation of the active component.

The effects of YashtimadhuKalpa on cognitive health need to be further studied and validated through pre-clinical and clinical studies. The longer shelf-life and enhanced palatability of the YashtimadhuKalpa is promising for its use as an alternative to currently used YashtimadhuChurna. The outcomes of pre-clinical and clinical studies will demonstrate the activity and role of YashtimadhuKalpa.

CONCLUSIONS

In this study, we presented the classic Ayurvedic Medhya Rasayan — Yashtimadhu Kalpa — in a simplified manner. The authenticity of the formulation was corroborated by HPTLC and HPLC. This study enforces the fact that the modification of classic Ayurvedic formulations is possible such that simplified and adapted formulations can be generated.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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REFERENCES


