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ORIGINAL ARTICLE

Comparative Study on Two Variants of *Laghupanchamula* (A Compound Ayurvedic Formulation) for Important Groups of Phytochemicals and Antioxidant Activity

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ABSTRACT

Introduction: *Laghupanchamula* is a compound formulation prepared by combinations of roots of five herbs. Two variants of *Laghupanchamula* have been described in Ayurvedic classics where beside four common herbs fifth one is either Gokshura (*Tribulus terrestris* L.) or Eranda (*Ricinus communis* L.). The objective of the study is to make comparison between two variants of Laghupanchmula with respect to important group of phytochemicals and antioxidant activity to corroborate the science behind their therapeutic utility.

Materials and methods: Standard methods have been followed for quantitative determination of total quantity of phenols, tannins, flavonoids, and flavonols, and *in vitro* antioxidant activity in variants of *Laghupanchmula* formulations. Qualitative high-performance liquid chromatography (HPLC) analysis has also been performed to establish presence/absence of important chemical constituents in formulations.

Results: Additional quantity of phenols, tannins, flavonoids, and flavonols has been observed in *Laghupanchamula* variant containing Eranda than the variant containing Gokshura. Greater antioxidant activity has also been found in formulation containing Eranda. The HPLC analysis revealed the presence of shikimic acid, gallic acid, catechin in both formulations, but rutin has been found only in formulation containing Eranda.

Conclusion: From the results of experiments it has been observed that the formulation containing Eranda has more antioxidant activity as it contains more quantities of phenol, tannins, flavonoids, and flavonols than the formulation containing Gokshura.

Keywords: Antioxidant activity, High-performance liquid chromatography profiling of Laghupanchmula, Laghupanchmula variants.

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INTRODUCTION

According to the World Health Organization, about 70 to 95% of developing countries of world rely on complementary, traditional, alternative, or nonconventional medicines for health care. Several traditional health care systems exist in India from centuries and out of all the traditional practices, Ayurveda, Yoga and Naturopathy, Unani, Siddha, and Homeopathy are the official traditional systems of medicine together known as Indian Systems of Medicine, which collectively provide health care to the vast majority of people of India and neighboring countries.[1]

Ayurveda is the science of longevity being practiced in India since 5000 BC as archaic system of traditional medicine, the term originating from the Sanskrit word "Ayus" and "Veda."[2,3] It is a comprehensive approach toward life, health, and disease management through medicinal herbs, minerals, diet, lifestyle, and spirituality. Ayurveda being pro-nature is developed over day-to-day experiences and collective relationship between people and nature, and thus not only cure diseases but also prevent diseases, helps in maintaining health, and promotes longevity.[4] Present-day lifestyle diseases are mainly reported as a result of imbalance between pro-oxidant and the antioxidant homeostatic phenomenon in the body. Oxidative conditions prevail mainly as a result of excessive release of the free radicals caused by undue oxidative stress, or due to the destitute suppression in the body caused by depletion of the dietary antioxidants.[5,6] The ancient Ayurvedic physicians inferred the exquisite body cellular system and the depreciation of the functional efficiency of the body tissues, hence they had a vision to develop certain dietary and therapeutic measures to arrest/delay ageing and rejuvenating whole functional dynamics of the body.

However, various plants are reported in present era for their promising antioxidant and free radical

scavenging activity. But there are very few studies on compound formulation of these plant drugs. *Laghupanchamula* is a compound formulation prepared by combinations of roots of five herbs. In a comprehensive review of Ayurvedic classics, it was revealed that there is description of two different variants of *Laghupanchamula* where besides four common herbs, viz. Kantakari (*Solanum surratense* Burm f.), Brihati (*Solanum indicum* L.), Shalaparni (*Desmodium gangeticum* DC.), and Prinshniparni (*Uraria picta* Desv.), the fifth one is either Gokshura (*Tribulus terrestris* L.) (LPG) or Eranda (*Ricinus communis* L.) (LPE).[7,8] These formulations have been documented for *Vataghna*, *Pittashamana*, *Brimghana*, and *Balavardhaka* properties.[9] This is a compound formulation, and the combination of whole herbs and their extracts containing complex mixture of phytochemicals may interact. Interactions between phytochemicals, and even between different plants used in combination, form the basis of therapeutic use in traditional healing paradigms of Ayurveda which may be attributed to the additive or synergistic activity of these phytoconstituents.[10] Therefore, a study was conducted on two groups of *Laghupanchamula* (LPG and LPE) to establish most suitable combination with greater antioxidant potential. Total phenolic, tannins, and flavonoids were quantified along with HPLC studies to know possible mechanism behind the activity and efficacy of two variants of *Laghupanchamula*. The aim of the study is to identify the difference in phytoconstituents of the two variants of *Laghupanchamula* which might be responsible for variation in pharmacological action of this important formulation.

MATERIALS AND METHODS

Chemical and Reagents

1,1-diphenyl-2-picryl-hydrazil (DPPH), shikmic acid, and catechin were procured from Sigma Aldrich. Sodium carbonate, sodium acetate, ferric chloride, rutin, ascorbic acid, gallic acid, Follin-Ciocalteu, 2-deoxyribose, thiobarbituric acid, and H_2O_2 (30%, v/v) were purchased from Merck India Ltd. or Qualigens Fine Chemical Co. (India). All other chemicals and solvents were of analytical grade.

Plant Material

Roots of *Laghupanchamula* plants were collected in the months of November–December 2011 from Rajiv Gandhi South Campus, Banaras Hindu University (BHU), Mirzapur, and authenticated by Prof VK Joshi, Department of Dravyaguna, Institute of Medical Sciences, BHU, India. Sample specimens (DG 1001–1006) were preserved in herbarium vide voucher in the Department of Dravyaguna for future reference.

Extraction Method

Equal weight (50 gm each) of dried root powder of Shalaparni, Prishniparni, Brihati, Kantakari, and Gokshura in first group (LPG), or Eranda in second group (LPE) was taken. Extracts of aforementioned two variants of *Laghupanchamula* were prepared separately by decoction using 50% ethanol following the standard procedures.[11] Yields of 10.9 and 10.8% were obtained for LPG and LPE respectively, by extraction method employed.

Quantitative Estimation of Phytoconstituents

The two variant formulations of *Laghupanchamula* (LPG and LPE) were subjected to estimation of various phytoconstituents. Total phenolic and tannin contents were estimated as per the method of Hagerman et al.[12] Standard methods were used to evaluate the total flavonoid and flavonol content.[13]

HPLC Analysis of Phenolic Compounds

The HPLC analyses of LPG and LPE were performed using HPLC system Shimadzu LC-10A (Japan) equipped with dual pump LC-10A binary system, ultraviolet (UV) detector SPD-10A, Phenomenex (Torrance, USA), and C18 column (RP-Hydro, 4 µm, 250 × 4.6 mm). Data were integrated employing Shimadzu class VP series software. Separation of compounds was achieved with acetonitrile/water (1:1 v/v) containing 1% acetic acid in a linear gradient program, started with 18% acetonitrile, changing to 32% in 15 minutes and finally to 50% in 40 minutes.[14] Solvent flow rate was maintained at 1.0 mL/minute. Results (mg/10 gm FW) were obtained by comparing the peak areas (λ_{max} = 254 nm) of the samples with those of standards (class VP series software, Shimadzu, Japan). Identification of the tested phenolic compounds was carried out by comparing their retention times and online UV spectra with those of available standards. Identified peaks were then confirmed by spiking samples with standard mixtures. Quantification was done by external standard method followed by integration of the peaks.

In vitro Antioxidant Activity

Free Radical Scavenging Activity using 1,1-diphenyl-2-picryl-hydrazil Method

The free radical scavenging activity of two variants of *Laghupanchamula* was evaluated by DPPH method. A solution of 100 µM/mL of DPPH in methanol was used

in this estimation. The absorbance of the reaction mixture was measured at 517 nm after 30 minutes.[15]

Nitric Oxide Scavenging Assay

Two variants of *Laghupanchamula* samples prepared in methanol were treated with sodium nitroprusside (10 mM) in phosphate buffered saline and incubated at room temperature for 150 minutes. The same reaction mixture without *Laghupanchamula* but the equivalent amount of methanol served as the control. Following the incubation time, 0.5 mL of Griess reagent was added. The absorbance was measured at 546 nm after 30 minutes.[16]

Scavenging of Hydrogen Peroxide

The standard method described by Jayaprakasha et al[17] was used for determining the scavenging activity of two variants of *Laghupanchamula*. The samples were treated with solution of hydrogen peroxide (20 mM) prepared in phosphate buffered saline, and finally the absorbance was measured at 230 nm after 10 minutes.

Scavenging of Hydroxyl Radical by Deoxyribose Method

For determining the hydroxyl radical scavenging activity of the two variants of *Laghupanchamula*, the variants were treated with 1 mM $FeCl_3$, 1 mM EDTA, 20 mM H_2O_2, 1 mM L-ascorbic acid, 30 mM deoxyribose, 1 mL of 2.8% (w/v) trichloroacetic acid, and 1 mL of 1% (w/w) 2-thiobarbituric acid in potassium phosphate buffer (pH 7.4). The reaction mixture was incubated for 1 hour at 37°C, and further heated in a boiling waterbath for 15 minutes after addition of 1 mL of 2.8% (w/v) trichloroacetic acid and 1 mL of 1% (w/w) 2-thiobarbituric acid. The color developed was measured at 532 nm against a blank containing phosphate buffer.[18]

RESULTS

Quantitative Estimation of Phytoconstituents

The results of quantification of phytoconstituents in two variants of Laghupanchmula are represented in Table 1. Total phenolic content quantified in LPE was found to be 117.4 ± 12.7 mg/gm tannic acid equivalent which was higher as compared with LPG 57.74 ± 8.1 mg/gm tannic acid equivalent, while total tannin determined in LPE was 96.52 ± 7.3 mg/gm tannic acid equivalent compared with 33.29 ± 7.2 mg/gm tannic acid equivalent in LPG. Total flavonoids and flavonols were found to be 84.15 ± 9.1 mg/gm and 15.26 ± 1.31 mg/gm rutin equivalent respectively, in LPE, whereas their quantity in LPG were found to be considerably lower as 46.53 ± 0.56 mg/gm and 2.01 ± 0.57 mg/gm rutin equivalent.

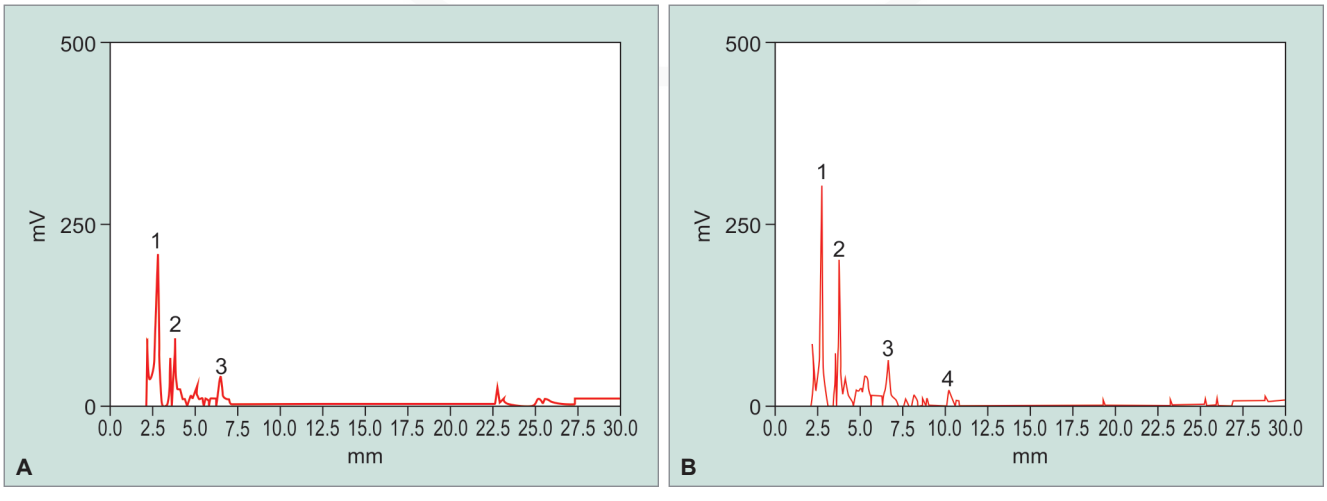
HPLC Analysis of Phenolic Compounds

Graphs 1A and B represent the HPLC chromatogram of LPG and LPE. Shikimic acid, catechin, gallic acid, and rutin were detected at 254 nm peaks. Shikimic acid, catechin, gallic acid, and rutin were detected in HPLC chromatogram of LPE, while rutin was absent in LPG. The content of various phenolic compounds detected in LPG and LPE are represented in Table 2.

Table 1: Quantification of phytoconstituents in two variants of *Laghupanchmula*

Phytoconstituents	*LPE*	*LPG*
Total phenolics (mg/gm TAE)	117.4 ± 12.7	57.74 ± 8.1
Total tannins (mg/gm TAE)	96.52 ± 7.3	33.29 ± 7.2
Total flavonoids (mg/gm RE)	84.15 ± 9.1	46.53 ± 0.56
Total flavonols (mg/gm RE)	15.26 ± 1.31	2.01 ± 0.57

TAE: Tannic acid equivalent; RE: Rutin equivalent



Graphs 1A and B: Chromatogram of phenolic compounds in two variants of *Laghupanchamula* at 254 nm. Peaks: 1: sikimic acid, 2: catechin, 3: gallic acid, 4: rutin: HPLC chromatogram of (A) LPG; and (B) LPE

Table 2: Phenolic compounds quantified in two variants of *Laghupanchmula* by HPLC

Standard	LPE (% w/w)	LPG (% w/w)
Shikimic acid	8.25	5.04
Catechin	6.15	2.70
Gallic acid	2.25	1.05
Rutin	2.00	–

In vitro Antioxidant Activity

Table 3 represents the results of the scavenging activity of DPPH, assay of nitric oxide, scavenging activity of hydrogen peroxide and hydroxyl radical for two variants of *Laghupanchamula*. The capability to reduce DPPH by donating an electron or hydrogen to DPPH is indicative of free radical scavenging activity of the extract. Both LPG and LPE in the present study demonstrated a considerable free radical scavenging activity as indicated by obtained IC_{50} values. The LPE showed an IC_{50} value of 38.15 ± 3.89 µg/mL, which is comparable to ascorbic acid (IC_{50}: 30.00 ± 1.28 µg/mL), while IC_{50} of LPG was found to be 54.19 ± 4.04. Griess reagent was used to determine the nitric oxide scavenging activity which illustrated a moderate scavenging activity of LPE (IC_{50}: 58.60 ± 3.85 µg/mL), whereas LPG showed IC_{50} of 74.74 ± 3.55 in comparison with ascorbic acid (IC_{50}: 9.71 ± 2.56 µg/mL). A considerably moderate scavenging potentials of hydrogen peroxide by LPE and LPG were observed with an IC_{50} value of 87.13 ± 4.21 and 117.4 ± 7.01 µg/mL respectively, as compared with standard ascorbic acid (IC_{50}: 57.50 ± 1.80 µg/mL). Fenton reaction was used to assess the potential of *Laghupanchamula* formulation in inhibiting the hydroxyl radical production through iron (II)–dependent deoxyribose damage assay. The results showed comparable scavenging activity of LPE (42.20 ± 4.84 µg/mL) with ascorbic acid (IC_{50} 22.91 ± 5.59 µg/mL), whereas LPG showed lower antioxidant activity with IC_{50} of 81.21 ± 4.73 µg/mL as compared with ascorbic acid and LPE.

DISCUSSION

Since earliest human civilizations in the Indian subcontinent, there is existence of Ayurveda. This traditional system of medicine of India has been evolved on the ground of practice and experience with nature and natural resources. There is a need of scientific evidence of claims of the Ayurvedic treatment in the light of the modern scientific knowledge and understanding, thereby making the system globally acceptable through unified approach which associates all together the traditional wisdom and modern scientific knowledge with expertise.

The results from quantitative estimation showed that the formulation was found to be rich in phenols, tannins, and flavonoids, however the *Laghupanchamula* containing Eranda showed presence of higher quantity of phenolics, tannins, and flavonoids as compared with the formulation containing Gokshura. The HPLC quantified presence of shikimic acid, catechin, gallic acid, and rutin in LPE, whereas rutin was absent in LPG. Phenolic compounds are main secondary metabolites, which include tannins and flavonoids. These are produced in plants serving variety of functions like defense against pathogens and different forms of environmental stress, e.g., heat stress, moisture stress, UV radiation.[19] Tannins and flavonoids also possess therapeutic uses due to their anti-inflammatory, antifungal, antioxidant, and healing properties.[20] Shikimic acid is an intermediate of the shikimic acid pathway[21] implicated in the synthesis of aromatic metabolites in plants and microorganisms.[22-25] Phenolic compounds, gallic acid and catechin, are widely found in plants. Gallic acid is a 3,4,5-trihydroxybenzoic acid found freely as well as part of hydrolyzable tannins, while catechin is a flavonoid belonging to flavan-3-ol group.[26] Rutin quantified in the present study is a flavonol glycoside that has been reported to have multiple pharmacological activities, such as antioxidant, cytoprotective, and wound-healing activity.[27,28] Various formulations used in Indian traditional medicine may act synergistically attributing to its antioxidant potential and thus preventing aging and related degenerative diseases. Free radicals are natural by-products of our own metabolism, which are electrically charged molecules that attack our cells, tearing through cellular membranes to react and create havoc with the nucleic acids, proteins, and

Table 3: *In vitro* antioxidant activity of two variants of *Laghupanchamula*

Drug	IC_{50} concentration (µg/mL) required for scavenging the free radical			
	DPPH radical	Nitric oxide scavenging	H_2O_2 radical	Hydroxyl radical
Standard				
Ascorbic acid	30.0 ± 1.28	13.71 ± 2.56	57.50 ± 1.80	22.91 ± 5.59
Extract				
LPE	38.15 ± 3.89	58.60 ± 3.85[a]	87.13 ± 4.21[a]	42.20 ± 4.84
LPG	54.19 ± 4.04[a]	74.74 ± 3.55[a]	117.4 ± 7.01[a]	81.21 ± 4.73[a]

All results are expressed as mean ± standard error of mean. Statistical comparison was determined by one-way analysis of variance followed by the Dunnett *post hoc* tests (comparison with control taken as standard ascorbic acid). [a]$p < 0.05$, statistically significant compared with ascorbic acid; Note: $p < 0.05$, statistical difference signifies lesser scavenging activity as compared with ascorbic acid

enzymes present in the body. Oxidative stress created by free radicals is capable of causing cells to lose their structure, function, and can eventually destroy them. Normally there is a balance between the amount of free radicals generated in the body and the antioxidant defense systems that scavenge/quench these free radicals preventing them from causing deleterious effects in the body, but increased load of free radicals in body, either due to environmental condition or produced within the body, results in oxidative stress, which may result in tissue injury and subsequent diseases.[29,30]

The study demonstrated that the *Laghupanchamula* formulation containing Eranda possessed more efficient antioxidant potential as compared with formulation containing Gokshura. Antioxidant phytoconstituents from medicinal plants have competence to abide oxidative stress by their ability to scavenge free radicals, impeding lipid peroxidation and various alternative methods. The antioxidative effectiveness of natural sources has been reported to be mostly due to presence of phenolic and flavonoid compounds. Polyphenolics play vital role in regulating body physiological mechanism, contributing to preventive measure in antioxidant, antimutagenic, and in diseases caused by oxidative stress. Hydroxyl group in phenolic compounds attribute to their scavenging ability. Flavonoids are a large group of ubiquitous molecules and possess antioxidant activities. Their planar structure, number, and position of their hydroxyl groups, as well as the presence of the C2–C3 double bond, are important for metal chelation, free radical scavenger capacities, and the inhibition of free radical–producing enzymes.[31] The present study found a correlation between the polyphenolic content and the antioxidant activity. It is also found that *Laghupanchamula* formulation containing Eranda showed higher quantity of phenolics and tannins and also found to contain rutin in addition to shikimic acid, gallic acid, and catechin as compared with *Laghupanchamula* formulation containing Gokshura.

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हिन्दी सारांश

फाइटोकेमिकल तथा एन्टी आक्सीडेंट क्रिया के महत्वपूर्ण समूह हेतु लघु पंचमूल (आयुर्वेदिक योग) के दो भेदों का तुलनात्मक अध्ययन

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परिचयः लघुपंचमूल पाँच औषधीय पादपों के मूल के संयोजन द्वारा तैयार यौगिक है। लघुपंचमूल के दो भेद आयुर्वेदिक शास्त्र में वर्णित किया गया है, जहां चार औषधीय पादपों के साथ में पांचवा या तो गोक्षुर (*Tribulas terrestris* L.) है या एरंड (*Ricinus communis* L.) है। अध्ययन का उद्देश्य फाइटोकेमिकल प्रोफाइल, तथा एंटीऑक्सीडेंट गतिविधि के संबंध में तुलनात्मक अध्ययन उनके चिकित्सीय उपयोगिता के पीछे विज्ञान की पुष्टि के लिए है।

पद्धतिः लघुपंचमूल के दो प्रकारों में पाया विभिन्न फाइटोकोंस्टीटूएंट्स का तुलनात्मक अध्ययन मात्रा और एचपीएलसी तरीकों द्वारा मूल्यांकन किया गया है। विभिन्न मॉडलों में इन विट्रो एंटीऑक्सीडेंट गतिविधि मूल्यांकन करने के लिए इस्तेमाल किया गया।

परिणामः गोक्षुर युक्त योग की तुलना में संम्पूर्ण फेनोलिक, टैनिन और फ्लवोनोइड्स की मात्रा एरंड युक्त लघुपंचमूल युक्त योग में मात्रा निर्धारित अधिक थी। एरंड युक्त एचपीएलसी में शिकिमिक एसिड, गैलिक एसिड, कैटेचिन और रूटीन उपस्थित था जबकि गोक्षुर युक्त में रूटीन अनुपस्थित था। इसके अलावा एरंड युक्त लघुपंचमूल में अधिक एंटीऑक्सीडेंट क्षमता थी।

चर्चाः अध्ययन का निष्कर्ष यह है कि एरंड युक्त लघुपंचमूल में ऐसे फेनोलिक और फ्लवोनोइड्स हैं जो अपनी उच्च एंटीऑक्सीडेंट क्षमता के लिए योगदान कर सकते हैं।

शब्द कुंजीः एंटीऑक्सीडेंट गतिविधि, लघुपंचमूल का एचपीएलसी, रूपरेखा, लघुपंचमूल प्रकार।