



Preliminary Phytochemical screening, *In vitro* antioxidant activity and acute oral toxicity study of polyherbal formulation for chronic kidney disease

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Abstract

Chronic Kidney Disease (CKD) is an increasing public health problem in developing countries with poor health outcomes. There is a constant demand to develop new, effective and affordable drugs for chronic kidney diseases (CKD). The Indian traditional medicinal system, Ayurveda is a valuable and alternative resource for identifying novel drugs for kidney diseases. The aim of the present study is to investigate the phytochemical screening, *in vitro* antioxidant activity, and *in vivo* acute oral toxicity study of polyherbal formulation for chronic kidney disease. All the experiments were conducted by using standard procedures. The results of the phytochemical screening revealed the presence of different types of secondary metabolites like alkaloids, phenols, saponins, flavonoids, steroids, tannins, and glycosides. The antioxidant activity of the formulation was evaluated by DPPH free radical scavenging assay. Rutin was used as a reference compound. The IC₅₀ value of the formulation was found to be 7.39071 % and the standard rutin was 5.69024 %. The acute toxicity study was conducted in Wistar albino rats. The formulation was administered as a single dose of 2000 mg/kg body weight. In this study, no visible behavioral changes and mortality was observed up to 2000 mg/kg body weight. We conclude that the polyherbal formulation did not exhibit any toxic effects on rats in terms of liver and renal functions, 15 days after administration. Taken together, these results provide scientific validation for the use of the polyherbal formulation as a potential treatment against chronic kidney disease.

Keywords: Polyherbal formulation, *Ayurveda*, Phytochemical screening, *in vitro* antioxidants, acute oral toxicity, albino rats.

1. INTRODUCTION

Chronic Kidney Disease (CKD) is a chief increasing public health problem in developing countries that has been related to poor health outcomes, decreased length and quality of life¹. The associated mortality and morbidity rate due to cardiovascular dysfunction, neurohumoral dysfunction and end stage renal disease can cost more than one trillion dollars in global clinical care annually. It is an internationally recognized public health problem affecting 5-10 % of the world population². The prevalence of CKD in India is at 13.8 % with over 90% of the patients unable to afford the cost. Early detection, valuation and protective management will be the key to delay progression and to prevent adverse outcomes. CKD is identified by measuring the levels of creatinine in blood, which is a breakdown product of muscle metabolism. Elevated creatinine levels indicate lower glomerular filtration rate and the result is a decrease in the capability of the kidneys to excrete waste products. Over one million people worldwide live on dialysis with a functioning graph indicating that the incidence of CKD has doubled in the last 15 years³. The latest management of CKD is not acceptable and the ultimate goal is renal transplant. It has become imperative for nephrologists and researchers to find suitable remedial measures from other alternative sources, Ayurveda being one of them. The management of diseases in Ayurveda is based on

its totalistic effect of drugs and measures with minimal side effects. Ayurveda declares that naming of disease is not necessary but the mainstay is to assess the Dosha, dushya, adhishtana along with strength of the disease and the patient, and then adopt appropriate therapeutic interventions. CKD is not fairly known in Ayurveda, but on the basis of the pathogenesis of the disease appropriate treatment measures are followed. Drugs combining the effect of Rookshana, Pachana and mutrala guna will be applicable in early stages with polyuric phase. Since Rasavaha srotomoola is Dasa dhamani (including adhogami mutravaha dhamani) CKD is a condition where rasa vaha sroto dushti chikitsa (That includes pachana) and rasayana (rejuvenative) principle also come into play. The drugs used in the polyherbal CKD formulation have been chosen on the basis of their traditional use as being tonic, aphrodisiac, palliative, astringent, lithotriptic, diuretic, hypouricemic, renal protective, antioxidant, anti-inflammatory, hepatoprotective, gastroprotective, antihypertensive, antidiabetic, antihyperlipidemic, and antimicrobial. A CKD-Renal care support polyherbal formulation was successfully developed at the Centre for Product Development, Heavenly Fuel P Ltd and was further studied for preliminary phytochemical screening, *in vitro* antioxidant and acute oral toxicity. Currently insufficient data exist on the utility of such a polyherbal formulation in CKD and

an attempt has been made through this paper to provide scientific evidence for its potential use in CKD.

2. PHARMACOLOGICAL EFFECT

2.1. Punarnava: (*Boerhavia diffusa*)

Ayurvedic concept: White variety of Punarnava has pungent taste with astringent secondary taste, cures anemia, stimulates appetite, reduces edema, vata, artificial poison, kapha, heals wounds and cures ascites. It has been included in the Guduchyadi Varga of Bhavaprakasha Nighantu.

Modern concept:

Studies have shown that the active phytochemicals in *Boerhavia diffusa* exhibit a number of pharmacological properties such as renoprotective or nephroprotective (Shikha et al., 2014) ⁴, antiurethritis (Pereira et al., 2009) ⁵, diuretic (Desai et al., 2008) ⁶.

2.2. Gokshura (*Tribulus terrestris*)

Ayurvedic concept:

Gokshura is sweet in taste, heavy to digest, unctuous, with a sweet post digestive taste. It is a coolant and balances all three doshas - Vata, Pitta and Kapa.

Modern concept

In India, *Tribulus terrestris* is used to treat kidney, liver, urinary and cardiovascular diseases. In addition, *Tribulus terrestris* was extensively used in Greek history to cure headache, nervous disruption, constipation and sexual dysfunction ⁷.

2.3. Pashanabheda (*Aerva lanata*)

Ayurvedic concept:

Pashanabheda is bitter - astringent in taste, light to digest, strong in action, with a pungent post digestive taste, hot in potency, breaks renal calculi and reduces Kapha vata.

Modern concept

In India it is commonly found in fields and waste places. The plant is extensively used in urinary disorders like urinary calculi, diuretic, used in lithiasis ⁸.

2.4. Shunti (*Zingiber officinale*)

Ayurvedic concept:

Shunti is laxative, heavy, penetrative in action, hot in potency, stimulates appetite, pungent in taste, has sweet post digestive taste, dry and reduces Vata- kapha.

Modern concept

Zingiber officinale is one of the most widely used herbs and flood flavouring agent and commonly known as ginger. The plant is reported for antimicrobial activity, anticancer activity, nephroprotective activity, anti-inflammatory activity ⁹.

2.5. Jeeraka (*Cuminum cyminum*)

Ayurvedic concept:

Jeeraka is dry, pungent, hot in potency, stimulates appetite, light to digest, reduces bowel movement, increases pitta, intelligence, cleanses uterine cavity, reduces fever, improves digestion, gives vigour, improves strength and taste, reduces kapha, promotes vision, cures bloating, gas, vomiting and diarrhea.

Modern concept

Cuminum cyminum was used to treat hoarseness, dyspepsia, jaundice and diarrhea. Its seeds were used for diuretic, carminative, astringent and stomachic ¹⁰. In India, cumin was used as an abortifacient, for kidney and bladder stones, chronic diarrhea ¹¹.

3. MATERIALS AND METHODS

3.1 Preparation and composition of the formula

The powdered form of the novel polyherbal formulation was prepared and manufactured according to AYUSH guidelines by Centre for Product Development, Heavenly Fuel P Ltd. It consisted of 5 herbs: Whole plant of Punarnava (*Boerhavia diffusa*), Fruit of Gokshura (*Tribulus terrestris*), Whole plant of Pashanabheda (*Aerva lanata*), Rhizome of Shunti (*Zingiber officinale*), Seeds of Jeeraka (*Cuminum cyminum*). All the plants were purchased from local market and were authenticated. The sample was washed under running water and was kept for shade drying. The dried sample was pulverized to a powder form. The ingredients were mixed thoroughly to obtain uniformity and stored in an airtight container.

3.2. Chemicals

Folin - Ciocalteu reagent, sodium carbonate, sodium nitrite, aluminium chloride, sodium hydroxide, DPPH (2,2-Diphenyl-1-picryl-hydrazyl), Rutin, Gallic acid, ferrous chloride, ferric chloride, Hydrochloric acid, sulphuric acid, sodium phosphate, ascorbic acid, were purchased from Hi-Media (Mumbai). All the chemicals and solvents used in this study were analytical grade.

3.3. Preliminary phytochemical analysis

The preliminary qualitative phytochemical screening of the poly herbal formulation was done to find out the different phytochemical constituents such as alkaloids, phenolic compounds, tannins, flavonoids, terpenoids, saponins, glycosides and steroids using standard methods ^{12,13}.

3.4. Determination of total phenolics

The quantitative estimation of phenolics in the polyherbal formulation was determined based on the standardized method ¹⁴. About 0.5ml of 1N Folin-Ciocalteu reagent and 2.5ml of 20% sodium carbonate solution were added sequentially in each tube containing different solvent extracts, followed by 40min dark incubation and the absorbance was recorded at 725nm against blank for the estimation of phenolics. The results were based on the calibration curve: $y = 0.025x - 0.056$, $R^2 = 0.998$ where x was the absorbance and y was the Gallic acid equivalents (mg/g) and were expressed in terms of milligrams Gallic acid equivalents (GAE) per gram of extract.

3.5. Determination of total flavonoids

Total flavonoid in the extracts is estimated by the general procedure ¹⁵. To each 300 μ l of polyherbal formulation 2ml of distilled water was added and followed by 150 μ l of NaNO_2 . The contents of the tubes were subjected to incubation for 6 min at room temperature. After incubation 150 μ l of AlCl_3 (10%) was added and incubated again for 6min at room temperature. Then 2ml of 4% NaOH was added, vortexed well and kept at room temperature for another 15min. The absorbance of pink color thereby developed was read spectrophotometrically at 510nm. The results were based on the calibration curve: $y = 0.002x - 0.008$, $R^2 = 0.996$ where x was the absorbance and y was the rutin equivalents (mg/g) and the results were expressed in terms of milligrams rutin equivalents per gram of extract.

3.6. *In vitro* antioxidant assays

The radical scavenging activity of the polyherbal formulation was determined by the standardized method of DPPH radical scavenging activity ¹⁶. A methanol solution of the sample extract at various concentrations was added to 5 ml of 0.1 mM methanolic solution of DPPH and allowed to stand for 20 min at 27°C. The absorbance of the solution was read spectrophotometrically at 517 nm. Methanol was served as blank and a solution without renal care support powder extract served as the negative control. The mixture of methanol, DPPH, and standard rutin served as positive control. The radical scavenging ability of the extract was expressed by the IC₅₀ value of the extract.

3.7. *In vivo* toxicity assay

The experiments were carried out on 8-9 weeks old healthy Wistar albino female rats, each weighing between 137 to 154 grams. The experimental procedures followed in this study were according to internationally acknowledged principles on laboratory animal use and care. The experimental procedures relating to the animals were duly approved by the University Animal Ethical Committee. Ethical clearance was obtained from the Institutional Animal Ethical Committee [JSS /IAEC /CADRAT, Dept of pharmacology]. All procedures were in strict compliance with relevant laws, the Animal Welfare Act, Public Health Services document, and guidelines established by the Institutional Animal Ethical Committee of the University.

3.8. Determination of acute oral toxicity

The six Wistar albino rats were kept in well-aerated polycarbonate cages under standard conditions of 23.4-28.8° under 12 hr light, and 12 hr dark cycle. The rats were housed in cages at random; selected ones were tagged and marked on the cages for identification. They were permitted to adapt to laboratory conditions for a week before starting the experiment. Drinking water and food (*Ad-libitum*) were provided during the experimental period. The acute oral toxicity of renal care support extract was evaluated in rats according to the procedures reviewed by the Organization for Economic Co-operation and Development [OECD] ¹⁷. The dose of 2000 mg/kg body weight of renal care support was administered orally to the rats in the treatment group. The dose limits were selected based on acute oral toxicity studies in rats, in conformity with the Organization in support of Economic Co-operation and Development [OECD] guidelines 423. The acute toxicity test was carried out in rats by repeated doses of 2000 mg/kg body weight. Food was provided to the rats approximately an hour after treatment. The rats were observed for any sign of toxicity effect for first 3 h, after the treatment period, up to 15 days. Visual observations on mortality, behavioral pattern, and changes in physical appearance, wound, ache, and signs of illness were monitored up to 15 days at 24 h interval.

4. RESULTS AND DISCUSSION

In the current study, the presence of alkaloids, flavonoids, phenolics, saponins, tannins, terpenoids, steroids, glycosides, proteins, amino acids and carbohydrates in the polyherbal formulation for chronic kidney disease was confirmed and is displayed in [Table 1]. Alkaloids are naturally occurring chemical compounds in plants that often have a wide range of pharmacological effects on diverse metabolic systems in humans and other animals. A phenolic compound shows antioxidant properties as oxygen scavengers, anti-bacterial, anti-viral, and anti-tumor properties ¹⁸. Tannins are high molecular weight phenolics that precipitate protein ¹⁹. Flavonoids are capable of scavenging oxygen derived free radicals and it possesses anti-viral and anti-inflammatory

properties ²⁰. Saponins are the bioactive compounds with both biological and pharmacological properties, which naturally occur in plants ²¹. Terpenoids are plant based compounds that have been traditionally used as a food source and have varied use in the chemical and pharmaceutical industries ²². Glycoside comes under the polyphenolic group that commonly occurs in plants which exhibit various anti-inflammatory properties ²³. Steroids have antibacterial properties that possess many medicinal and pharmaceutical activities to enhance the immune response ²⁴.

Table 1: Phytochemical screening of Polyherbal Formulation

S.No	Phytochemical constituents	Presence or absence
1	Alkaloids	++
2	Phenolic compounds	+++
3	Tannins	++
4	Flavonoids	++
5	Terpenoids	+
6	Steroids	++
7	Glycosides	++
8	Flavanol glycosides	+
9	Cardiac glycosides	+
10	Saponins	+
11	Phytosterol	+
12	Fixed oils and fats	+
13	Carbohydrates	++
14	Proteins	+
15	Amino acids	+

(+): presence of chemicals, (-): absence of chemicals or not detectable concentration, (+) < (++) < (+++): based on the intensity of characteristic.

4.1. Determination of total phenolics and flavonoid contents

The quantitative phytochemical screening of total phenolics and flavonoids content was analyzed in the polyherbal formulation of chronic kidney disease shown in the [Table 2]. Total phenolic content of polyherbal formulation showed the content value of 424.7651 %. The total flavonoid content of polyherbal formulation showed the content value of 40.85641%. Total phenolic content was found higher than flavonoid in the polyherbal formulation of chronic kidney disease. Phenolic compounds play a significant role in antioxidant activity as well as an important biological function of the plant ²⁵. Flavonoids and tannins are the phenolic compounds that belong to the chief group of plant phenolics; they are free radical scavengers and are primary antioxidants ²⁶. Since these compounds were found to be present in polyherbal formulation it might be responsible for the potent antioxidant capacity in scavenging the free radicals. Pharmacological properties of polyphenols are found in the renal area, acting as diuretic, anti-inflammatory, antispasmodic and antioxidant agents. Various polyphenolic compounds have been reported for their nephroprotective activity with a good level of renal protection. Therefore, considering the important role of polyphenolic compounds in the prevention or reduction of renal disorders induced by various nephrotoxic chemical agents and some antioxidant plants such as, *Terminalia chebula* and *Zingiber officinalis* having Nephroprotective properties ²⁷.

Table 2: Quantitative phytochemical analysis of Polyherbal Formulation

Quantitative phytochemical analysis	
Phytoconstituents	Percentage (%)
Phenols	424.7651
Flavonoids	40.85641

4.2. *In vitro* antioxidant assay

Antioxidants are the organic constituents, are highly used over natural sources, which are also a mixture of phytochemicals. The strong generation of oxidative stress by prooxidants damages the cellular molecules such as proteins, nucleic acids, and lipids which may cause tissue interruption ²⁸. In such conditions, antioxidants perform a vital role in the elimination of oxidative stress by scavenging the radicals causing oxidative damage. Therefore, the antioxidants found in the poly herbal formulation can play a vital role in scavenging the free radicals. This study was aimed to evaluate the antioxidant capacity of the polyherbal formulation to identify its ability to scavenge the free radicals and the results are reported in [Table 3]. The DPPH scavenging assay was used to study the free radical scavenging activity of the extract by calculating the IC₅₀ (half maximal inhibitory concentration) values of the extract. Lower IC₅₀

value of poly herbal formulation reflects higher DPPH radical scavenging activity. It can be concluded that the poly herbal formulation showed more potent *in vitro* antioxidant activity in dose dependent manner with higher percentage of inhibition at 50 mcg/ml. The IC₅₀ value of poly herbal formulation was found to be 7.390719 % antioxidant activity of this poly herbal formulation might be due to the presence of higher amount of phenolic compounds. Generally the phenolic compounds are considered as primary antioxidant.

Table 3: Determination of DPPH scavenging activity

Extract	DPPH radical scavenging activity IC ₅₀ value (µg/ml)
Polyherbal Formulation	7.390719 %
Rutin (control)	5.69024 %

4.3. *In vivo* toxicity study

Acute oral toxicity test was performed at 2000 mg/kg body weight post orally as a single dose for 15 days. The body weight of the control and poly herbal formulation treated groups were increased progressively throughout the experimental period as shown in [Table 4].

Table 4: Body Weight, Body Weight Changes and Pre-Terminal Deaths

Dose (mg/kg b.wt.)	Rat No.	Sex	Body weight (g)					No. dead / No. tested
			Initial	Day 8	Weight change (day 8 - Initial)	Day 15	Weight change (day 15 - Initial)	
2000	R001	Female	149	158	9	164	15	0/6
	R002	Female	146	151	5	154	8	
	R003	Female	139	143	4	150	11	
	R004	Female	143	148	5	157	14	
	R005	Female	152	161	9	168	16	
	R006	Female	141	146	5	152	11	

No toxic signs and necropsy findings were observed in [Table 5]. On day one, after the administration of doses the animals were kept under observation for the first 4 hours and then on a daily basis then other parameters like fur, skin, breathing, salivation, hair fall etc were observed. Any changes or abnormalities recorded could be an indication of toxicity. The test animals at all dose levels of poly herbal formulations showed no significant changes in behavior before and after treatment. Body weight change is an important factor to monitor animal health. Any loss in body weight is frequently

the first indicator of onset of an adverse effect. A dose, which causes 10 % or more reduction in the body weight is considered a toxic dose. All treated animals showed a 10 to 15 gram increase in body weight at the end of the 15th day as compared to initial observations. There was no mortality recorded at the end of the study, even at the fixed dose of 2000 mg/kg body weight of poly herbal formulations. At the end of the observation period, the rats were sacrificed by using diethyl ether anesthesia and subjected to detailed necropsy.

Table 5: Individual Toxic Signs and Necropsy Findings

Rat No.	Fasting body weight	Dose volume (ml)	Day of observation																			Necropsy findings
			Day 1					2	3	4	5	6	7	8	9	10	11	12	13	14	15	
			30 min.	1 hr	2 hr	3 hr	4 hr															
R001	143	1.4	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	NAD
R002	140	1.4	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	NAD
R003	130	1.3	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	NAD
R004	135	1.4	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	NAD
R005	146	1.5	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	NAD
R006	134	1.3	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	001	NAD

Abbreviations: F: Female; Min. Minute; hr: Hour; NAD: No abnormality detected; 001: No specific findings.

5. CONCLUSION

Phytochemical constituent of poly herbal formulation of chronic kidney disease revealed the presence of alkaloids, phenols, saponins, flavonoids, steroids, glycosides, proteins, amino acids and carbohydrates. The high phenolic content points to a high antioxidant potential. Our results showed the presence of various phytochemicals in the poly herbal formulation, which may be responsible for its pharmacological properties of the extract. Various medicinal plants have been reported to exhibit toxicity. Hence a Preliminary toxicological evaluation was carried out for the authentication of safety of the herbal medicine. During the course of the experiment significant difference was observed in the body weight of the rats treated with poly herbal formulation. No toxicity or mortality was observed during and after the experimental period. The medicinal plants should have low toxicity because of their long-term use in humans. Further long term efficacy and nephrotoxicity studies using the CKD poly herbal formulation may therefore be warranted.

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AUTHORS CONTRIBUTIONS

Each author has given considerable and equal contributions to this research

CONFLICTS OF INTEREST

The authors have given considerable and equal contributions to this research.

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