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Research Article

Acute and Subchronic Toxicity and LC-MS Fingerprinting of a Polyherbal Formulation (KNDBHU) used for COVID-19 Management

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ABSTRACT

A polyherbal formulation consisting of *Withania somnifera, Tinospora cordifolia, Moringa oleifera, Adhatoda vasica, Pipper longum, Glycyrrhiza glabra, Ocimum sanctum* and *Curcuma longa* has been used for Covid-19 treatment empirically, which was found effective. However, toxicity data were not available for this polyherbal formulation. This study aims to assess the polyherbal formulation's oral acute and subchronic toxicity in rats. The fixed-dose approach was used to conduct the acute toxicity investigation on 6 female Wistar rats for the treatment group and 5 female Wistar rats for the control group. A single dosage of this polyherbal formulation weighing 2,000 mg/kg was administered orally to the test group. At the end of the investigation, no fatalities or major toxic effects were noted, and it was determined that the lethal dose 50% (LD50) of the polyherbal formulation was greater than 2,000 mg/kg. Vital organs underwent macroscopic and microscopic inspection, neither of which revealed any toxicity signs. The polyherbal formulation was given orally for 91 days during the subchronic toxicity research in dose variations: 250 mg/kg, 500 mg/kg, 1,000 mg/kg, 2,000 mg/kg, and 4,000 mg/kg. The daily dose for a human is the same at the lowest level of 250 mg/kg. On physical signs and symptoms, weight growth, food intake, haematological parameters, biochemical parameters, and macroscopic and microscopic examination of organs, no major harmful effects were seen at any of these doses. These results demonstrated that the oral administration of this polyherbal formulation over the short- and long-term is safe when taken as suggested.

Keywords: Ayurvedic; Polyherbal; COVID19; Acute toxicity; Subchronic Oral Toxicity

INTRODUCTION

The coronaviruses (CoVs) are members of the order Nidovirales, family Corona viridae, and genus Coronavirus. The SARS-CoV-2 virus has been responsible for the Coronavirus disease (COVID-19) and its related pandemic for the past three years. Since coronaviruses are zoonotic in nature, they can spread to people and have in the past caused numerous epidemics. A respiratory infection is brought on by beta-coronavirus, a single-stranded positive sense RNA virus from the Corona viridea family with spike-like projections. Prior to the current pandemic, common coronavirus strains that infected people were NL63, OC43, 229E, and HKU1. Three novel coronavirus strains, SARS CoV, MERS CoV, and SARS-CoV-2, have emerged over the past ten years with rising morbidity and death. [1]

Wuhan, China, reported cases of acute respiratory distress in the latter part of 2019. On March 11, 2020, the World Health Organization (WHO) declared COVID-19's subsequent breakout to be a pandemic. The pandemic epidemic, which has spread to 200 nations and affected millions of people, is one of the major health concerns of the decade. Worldwide pharmaceutical companies have been hunting for an effective antiviral treatment for this virus. To treat COVID-19, drugs such monoclonal antibodies, antisense RNA, corticosteroids, and convalescent plasma have all been "repurposed," along with chloroquine, hydroxychloroquine, favipiravir, and others.The main treatments for managing COVID-19 are these medications and supportive therapies such oxygen breathing and hydration control. Remdesivir, a well-known antiviral medication for RNA viruses, has been used widely in COVID-19 patients among them. However, there are several significant drawbacks to repurposing antivirals, including the absence of a therapeutic cure, adverse effects, a lack of dose trials, and the formation of

mutant strains. There is an urgent need for an antiviral herbal formulation that can attack the virus specifically and have less adverse effect.[2]

Medicinal plants have abundance of phytochemical which have potency to inhibit the viral infection including COVID-19. Due to the presence of alkaloids, flavonoids, tannins, phenols, and other phytochemicals, herbal formulations might have strong antiviral activities.[3] The present study focuses on a novel Ayurvedic formulation, KNDBHU which contains 8 ingredients *Withania somnifera, Tinospora cordifolia, Moringa oleifera, Adhatoda vasica, Pipper longum, Glycyrrhiza glabra, Ocimum sanctum* and *Curcuma longa* in powder form and its extract. In India, the home of Ayurveda, some of these plants are frequently taken due to their flavour and other health advantages. These herbs also serve as food preservative due to their high antibacterial activity. *Withania somnifera* (Ashwagandha) contains a number of bioactive substances, including Withanoside V, Withanone, Somniferine, and others, which may be useful in the management and treatment of COVID-19 infection. It may help to keep the body's immune system in balance, regulate inflammation, reduce pro-inflammatory cytokines, protect various organs, and have antiviral, anti-stress, and antihypertensive properties.[4] Amritoside and Apigenin-6-C-glucosyl7-O-glucoside from *Tinospora cordifolia* (Guduchi) demonstrated the strong affinity to COVID-19 therapy.[5] *Moringa oleifera* (Sahjan) contains kaempferol 3-O-neohesperidoside, 3-L-dirhamnosyl-(1-4)-D-glucopyranoside, and quercetin 3-O-glucoside as principal constituents which revealed antiviral property.[6] *Adhatoda vasica* (Vasak) contents vasicine, vasicinone, vasicinolone, vasicol, and vasicolinone, together with aniflorine, anisotine, vasnetine, and orientin which has antiviral activity.[2] Vicenin, piperine, anthraglycosides, sterols, alkaloids, and a number of other substances found in *Piper longum* (Pipali) have shown a great potential for COVID-19 therapy.[7] *Glycyrrhiza glabra* (Yashtimadhu) has 3,R-glabridin, 7,4′-dihydroxyflavone, formononetin, isoliquiritigenin, liquiritin, naringenin, Liquiritin apioside, 3,3,4,4-tetrahydroxy-2-methoxychalcone, isoliquiritigenin-4′-O-d-apiosylglucoside, and isoliquiritigenin-4-O-d-apiosylglucoside and 5-O-glucoside which are promising antivirals, anti-inflammatory and immunomodulators.[8] The compounds eugenol (1-hydroxy-2-methoxy-4-allylbenzene), euginal, urosolic acid (2,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydro-1H-picene-4a-carboxylic acid), carvacrol (5-isopropyl-2-methylphenol), linalool (3,7-dimethyloct .0] methyl undec-4-ene, also known as estragole (1-allyl-4-methoxybenzene) are found in *Ocimum sanctum* (Tulsi) which are also promising antivirals, anti-inflammatory and immunomodulators.[9] Major component of *Curcuma longa* (Haridra) is curcumin which is able to influence a number of molecular targets, including transcriptional regulators (NF-B, Nrf2, AP-1, STAT-1,-3,-4), enzymes (COX-2, iNOS, OH-1, LOX, XO), inflammatory cytokines (chemokine ligand, interleukin, TNF), proteins (caspase-3,-9, Bcl-2, prostaglandin, CRP, myosin light chain), protein kinase (AK, JNK, JAK, MAPK), receptors (TLRs, chemokine receptors, TGF-, TGF-ß) and growth factors.[10] The formulation prepared (KNDBHU) using these medicinal plants in this study reportedly exhibit potential therapeutic effect in the COVID-19 patients. The present study aims to evaluate toxicity for clinical use.

METHODOLOGY

Preparation of Polyherbal Formulation

This polyherbal formulation was prepared with 8 well known medicinal plants, namely *Withania somnifera, Tinospora cordifolia, Moringa oleifera, Adhatoda vasica, Pipper longum, Glycyrrhiza glabra, Ocimum sanctum* and *Curcuma longa* in equal amount. The drugs were powdered with domestic grinder machine and packed for human clinical trial, and extracted with hydroalcohol (60:40) for animal toxicity studies. The images of raw drugs are shown below Figure 1

Fig. 1: Components of the polyherbal formulation (KNDBHU). *Withania somnifera* (root), *Tinospora cordifolia* (stem), *Moringa oleifera* (bark), *Adhatoda vasica* (all parts), *Pipper longum* (spike), *Glycyrrhiza glabra* (stem), *Ocimum sanctum* (leaves) and *Curcuma longa* (rhizome)

Extraction and Fractionation

The powder formulated drug was extracted with 60% hydro-alcohol solvent through maceration and further fractionated with column chromatography using non-polar solvent to polar solvent, hexane, chloroform, ethyl acetate, and water respectively. The factions were concentrated with rotary evaporator and further dried in vacuum oven. The samples were coded as AH, AC, AE, and AW as per the solvents.

LC-MS finger printing

LC-MS analysis for finger printing of the fraction of the polyherbal formulation was carried out using Acetonitrile and 5mM $AcNH_4$ buffer, pH 6.5 as solvents through C18 (250X4.6, 5μm) column (Waters Alliance e2695/HPLC-TQD Mass spectrometer at Sophisticated Analytical Instrument facility, CSIR-Central Drug Research Institute, Lucknow). The scan range was 150 to 2000. Mass by charge ration (m/z) of fractions of the formulation were employed in LC-MS for comparative fingerprint profiling of various chemicals found in individual fraction. [11]

Experimental Animals

The central animal house, Institute of Medical Sciences, Banaras Hindu University, Varanasi provided the Wistar rats for this study. The animals were kept in cages with wire net coverings in a room with a 12 h light/dark cycle, 70% humidity, 25°C temperature, and enough ventilation. All animals had unlimited access to food and water. The Animal Ethical Committee of the institute (542/GO/ReBi//S/02/CPCSEA/dated 26.5.2017) had approved the experiments and mandated that they be carried out in accordance with the generally recognised standards for the use and care of animals in laboratories.

Acute Toxicity Study

The acute toxicity test was carried out in accordance with OECD guideline 420 using a fixed-dose technique. According to OECD recommendations, testing on one sex typically females is sufficient, therefore six female Wistar rats, aged 8 to 10 weeks, weighing around 150g, had a 7-day acclimatization period. In this study, there were no in vivo and in vitro toxicity data for the polyherbal formulation, the initial dose was set at 300 mg/kg. Since we did not see any toxicity signs in the initial research on one rat with a dose of 300 mg/kg, the dose was increased to 2,000 mg/kg. Additional preliminary studies at a level of 2,000 mg/kg likewise revealed that no hazardous effects were present. A dose of 2,000 mg/kg was also administered to 4 additional rats in the first test. Within the first 24 hours, each animal was watched and its poisoning signs were noted. After that, observation was continued for another 14 days. [12]

Sub-chronic Toxicity Study

The OECD guideline 407 for evaluating compounds was followed for conducting the sub-chronic toxicity test. Sixty Wistar rats, 5 males and 5 females, aged 10 weeks, were kept into 5 treatment groups and 1 control group, totaling 6 groups (n = 10). Polyherbal formulations containing 250, 500, 1000, 2000 and 4000 mg/kg were given to the treatment groups, respectively. 10 ml/kg of water was administered to the control group. The polyherbal formulation or water was administered orally once daily between 9:00 and 10:00 am for 91 days. The rats were examined for any physical indications or symptoms of poisoning. Weekly calculations were made of food intake and weight growth. After 91 days of the study, a macroscopic and histological evaluation of the vital organs was carried out. After the study, during scarification the blood was collected from heart of the rats and serum was analyzed for biochemical variation in which SGOT, SGPT, and Creatinine were estimated as blood parameters. The data were recorded as mean ±SD. One-way ANOVA was used to assess the mean difference between all groups. [12]

RESULTS

LC-MS finger printing

The finger printing developed by LC-MS is most sensitive and may be used for the standardization of herbal drugs or formulations. There were four fractions analyzed for the fingerprinting that showed distinguished pattern. The hexane fraction of hydro-alcoholic extract of KNDBHU (AH) revealed m/z peaks at 165.0201, 231.1403, 255.2348, 271.2294, 279.2353, 295.2302, 311.2252, 325.1510, 351.2246, 375.2235, 389.2477, 455.3573, 471.3478, 523.3436, 551.3462 and 600.3945; AC showed peaks at 193.0512, 285.0426, 307.0999, 337.1101, 387.1187, 425.1038, 481.2586, 567.2318, 623.2428, 687.2320, 982.9913 and 1033.9879; AE showed peak at 187.0984, 225.1132, 311.1156, 347.0940, 525.3107, 555.2880, 643.2333, 687.2373, 747.2596, 982.9906 and 1033.9882. The details of the peaks are given in Figure 2.

Acute toxicity study

All test animals for acute toxicity were closely monitored for the appearance of any toxic signs or symptoms at intervals of 0, 30 min, 1, 2, 4, 6, 8, 12, and 24 h, as well as every day for 14 days. During the acute toxicity assessment, no hazardous symptoms were seen in the clinical parameters. Therefore, it suggests that the polyherbal formulation's LD50 is higher than 2000 mg/kg/day (Table 1).

Sub-acute toxicity study

The completion of the study was finalized with histopathological examination of vital organs and biochemical evaluation. The slides of control (Figure 3 A) and treated (Figure 3 B) group of rats revealed identical cellular architecture. Similarly, the liver of rats is also histopathologically similar in cellular architecture in both control (Figure 3 C) and treated (Figure 3 D) group of rats. There is no change found in slides of lung (Figure 3 E & F) and brain (Figure 3 G & H) of the rats in treated and control group. The blood serum parameters, SGOT, SGPT, and Creatinine revealed no significant change in control and treated groups of the rats (Table 2).

Table 1: Morphological and behavioral observation in acute toxicity study

Observation	0 h		0.5 h		1 h		2 h		4 h		24 h		14th day	
	D1	D2	D1	D2	D1	D2	D1	D2	D1	D2	D1	D2	D1	D2
Skin & fur	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Mucous membrane	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Tremors	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Convulsion	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Salivation	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Diarrhea	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Sleep	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Coma	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Lethargy	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Mortality	N	N	N	N	N	N	N	N	N	N	N	N	N	N

Table 2: Biochemical variation between control and treated group of rats

Treatment	Dose(ml/kg)	SGOT(U/L)	SGPT(U/L)	Creatinine(mg/dl)
Normal control	water	148.05±6.45	91.45±2.53	051±0.06
Treated group	4,000 mg/kg b.wt	145.26±4.26	91.51±5.34	0.52±0.04

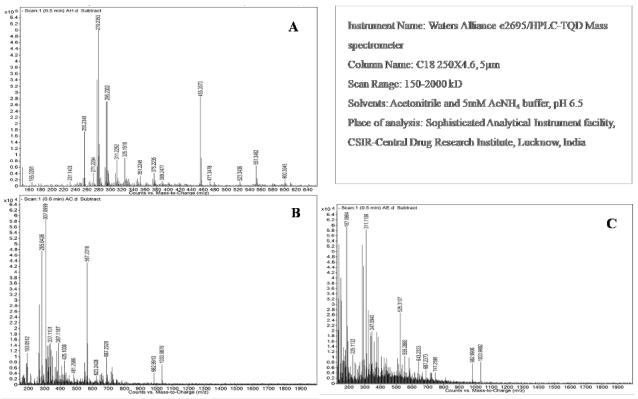

Fig. 2: Liquid Chromatography-Mass Spectroscopic fingerprinting data of A: AH, B: AC, and C: AE of KNDBHU polyherbal drug formulation

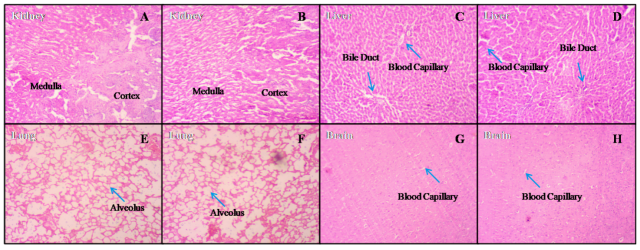

Fig. 3: Kidney, Liver, Lung & Brain Section of Rat (Control) and (Treated)X100

DISCUSION

For the past decade, the best approach for separating, characterizing, and identifying the active components of herbal products has emerged as liquid chromatography-mass spectrometry. This technique has also had a considerable impact on drug development and standardization. The fingerprint developed through LC-MS is considered as most precise method. Its accuracy and dependability is well established, and the fingerprint developed by it is analyzed for polyherbal drug quality control. This technique also aids in identification and verification while preventing adulteration.[11] In this study, the distinct peaks of the fractions establish the base line for standardization of the formulation as given in Figure 2. The three fractions of Hydro-alcoholic extract of KNDBHU have distinct peaks such as Hexane fraction showed m/z peaks at 165.0201, 231.1403, 255.2348, 271.2294, 279.2353, 295.2302, 311.2252, 325.1510, 351.2246, 375.2235, 389.2477, 455.3573, 471.3478, 523.3436, 551.3462 and 600.3945; Chloroform fraction showed peaks at 193.0512, 285.0426, 307.0999, 337.1101, 387.1187, 425.1038, 481.2586, 567.2318, 623.2428, 687.2320, 982.9913 and 1033.9879, and Ethylacetate fraction showed peak at 187.0984, 225.1132, 311.1156, 347.0940, 525.3107, 555.2880, 643.2333, 687.2373, 747.2596, 982.9906 and 1033.9882. Comparing the allopathic therapy, polyherbal formulations are frequently employed to treat many ailments in industrialised nations.[13] In this study, rats were used to investigate the acute and sub-acute toxicity of herbal products. At a dosage of 2000 mg/kg/day for acute toxicity investigation, no morbidity or mortality was recorded. This demonstrated that a dose of 2000 mg/kg/day of the medication can be safely used for acute therapy and biochemical markers of the liver, and renal markers were

seen during the sub-acute toxicity study, indicating that these doses could be used safely for the treatment of COVID-19 patients. The formulation includes ingredients namely, *Withania somnifera, Tinospora cordifolia, Moringa oleifera, Adhatoda vasica, Pipper longum, Glycyrrhiza glabra, Ocimum sanctum* and *Curcuma longa* in equal amount that have been linked to reducing inflammation, have antiviral, antibacterial, antioxidant activities. These medications have reportedly been shown to include various phytochemicals, making them demonstrate anti-inflammatory effects and chemotactic cytokines (TNF and IL-6).[14] At a dose of 50 mg/kg/day, there were no changes in the morphology of the liver or kidneys noticed during the histopathological examination. Although no change in the biochemical parameters was seen during the sub-acute research, the rats were shown to have significantly lower levels of the liver enzymes throughout the sub-acute toxicity trials when the dose was 200 mg/kg/day, which was below the normal reference range.

CONCLUSIONS

The results of the acute investigation showed that a single oral dose of 2000 mg/kg/day of this polyherbal formulation is not harmful. The 28-day subacute toxicity trial with 50 mg/kg/day found no significant alterations. In light of this, it can be said that the formulation is safe for a period of 28 days.

FURTHER RESEARCH

It is advised to examine the effect of this combination on other organs and tissues after higher dose administration for a long time to assess any probable toxic risk from this polyherbal formulation.

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CONFLICT OF INTERES T

The authors are declared that there is no conflict of interest.

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