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THE PHYSICOCHEMICAL, PHYTOCHEMICAL, AND *IN-VITRO* SAFETY STUDY OF KAYAM CHURNA

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ABSTRACT

Kayam churna (KC) is an Indian Polyherbal formulation, a mixture of herbs. For generations, people have used the polyherbal remedy Kayam churna to treat conditions including diarrhea and constipation, improve digestion, treat ulcers, and more. It is said to be effective and safe. The current study aimed to investigate the presence of heavy metals, phytochemical analysis, and also in-vitro studies on HepG2 cell line to investigate the anti-oxidant and cytotoxic potentials of Kayam churna considering that they are utilized by a significant proportion of the population in India. The heavy metal analysis was done by Atomic Absorption Spectroscopy (AAS), phytochemical analysis by standard methods, and cytotoxicity assessment by MTT assay with five different concentrations of drug (62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, and 1000 µg/ml) and anti-oxidant study by the evaluation of ROS level by Anti-ROS assay (H_2DCFDA Staining method). The AAS study of the test drug revealed the very low concentration (below the permissible limit) of heavy like cadmium, lead, zinc, and copper. The Phytochemical analysis showed the presence of carbohydrates, glycosides, saponins, flavonoids, and steroids. When the HepG2 cell line was exposed to higher concentrations (1000 g/ml) of the test drug, no noticeable reduction in cell viability was observed. Also, the anti-oxidant study of KC indicates that the antioxidant defense system in cells is successfully lowering oxidative stress. Therefore, the tested Ayurvedic formulation (Kayam-churna) was found to be safe, lacking significant liver cytotoxicity, having a potential anti-oxidant effect, and did not contain heavy metal concentrations over the permissible limits.

Keywords; Kayam Churna, AAS, MTT assay, H_2DCFDA Staining method, Flow cytometry

INTRODUCTION

Traditional medicine is used by more than 75% of the world's population for basic health conditions. India, the epicenter of Ayurvedic medical system, is home to more than 45,000 plant species out of these, 7500 plant species are found praxis in convalesce. An ancient Indian traditional medical system; Ayurveda has been used for thousands of years, where various natural remedies, including plants, animals, and minerals derivatives are used to treat human illnesses **[1]**. It is based on the idea that health and wellness depend on a delicate balance between the mind, body, and spirit. Ayurveda emphasizes the importance of maintaining this balance to promote good health, prevent illness, and treat diseases. Many proponents of Ayurvedic remedies support that if Ayurvedic preparations are appropriately used by following the standard therapeutic doses, the formulations with a long history of their uses are generally safe **[2]**. Also, compared to chemical treatments, several natural formulations that contain plant extracts have been discovered to be safer medications with fewer adverse effects **[3]**. Nonetheless, while accepting the value of Ayurveda, the modern pharmaceutical industries are leading today to the production of numerous Ayurvedic formulations using natural ingredients to develop potent medications for a variety of ailments **[4, 5]** like Yakrit plihantak churna for liver disease[6], Pancharishta for stomach ulcers, and hepatitis **[7]**, Triphala for appetite stimulation, reduction of hyperacidity, etc. **[8]**, Arogyavardhini vati for jaundice, liver disease and several skin related disorders **[9]**. There are plenty of polyherbal formulations used successfully in the Ayurvedic system, but most of them are struggling with a lack of evidence on their precise mechanism of action. Such one popularly used formulation is Kayam churna.

Kayam-churna is a mixture of herbs namely *Cassia angustifolia* (Senna leaves), *Opervulina trupethum* (Nishoth), *Terminalia chebula* (Haritaki), *Glycyrrhiza glabra* (Mulethi) used in the treatment of constipation, improve digestion, help to relief in acidity, etc. **[10]**. The demand for Ayurvedic preparations has increased globally due to their natural origin and lack of negative effects. Due to the limited availability and high expense of traditional, authentic medicines, the commercialization of traditional medicines has resulted in the widespread use of adulterants and low-priced alternatives. Therefore, it is now essential to verify polyherbal formulations by modern research guidelines to standardize and assess their quality, safety, and efficacy. Therefore, to assess and validate the therapeutic benefits of Ayurvedic formulations, the current study

aimed to evaluate the physicochemical, phytochemical, in-vitro cytotoxic effects, and antioxidant study of Kayam-churna. The physicochemical studies were carried out to make sure that the Ayurvedic formulation is safe for ingestion and does not include excessive levels of toxic metals like lead, chromium, and cadmium. The plant-based substances used in traditional Ayurvedic formulations are frequently collected from throughout the world and may occasionally be contaminated with heavy metals from the soil, water, or air. And when heavy metals accumulated in the body over time, they can result in major health issues like cancer, kidney impairment, and neurological problems. Therefore, to assure the safety and purity of these Ayurvedic formulations and protect customers from harmful exposure to toxic metals, heavy metals analysis of Ayurvedic formulations is important. So for this, the atomic absorption spectroscopic method was used. The presence of phytochemicals in Ayurvedic formulation is also responsible for its therapeutic effect. Therefore, the phytochemical analysis can also assist in determining any impurities or adulterants in the formulations that might compromise the final product's quality and safety. Additionally the biological safety assessment of Ayurvedic formulations was carried out by the cytotoxicity assessment. This is essential since many Ayurvedic formulations contain a variety of natural ingredients, some of which may be harmful when used in large doses or high concentrations. The cytotoxicity assessment of Ayurvedic formulations can assist in identifying potentially harmful chemicals and determining safe concentrations of these substances for use in medicine. Additionally, it can be useful in ensuring that Ayurvedic formulations are safe for human consumption. Overall, the evaluation of cytotoxicity is crucial that ensures the safety and effectiveness of Ayurvedic formulations. Therefore, for the cytotoxicity assessment of Ayurvedic formulation liver cell lines are the first approach to conducting studies because the liver is a vital organ that is crucial to the metabolism and detoxification of drugs. Liver cells, sometimes referred to as Hepatocytes, are the body's main location for drug metabolism. Also, for the more physiologically justified and biologically appropriate assessment of potential therapeutic effects on diseases linked to oxidative stress, assessment of anti-ROS activity of Ayurvedic formulations are very important.

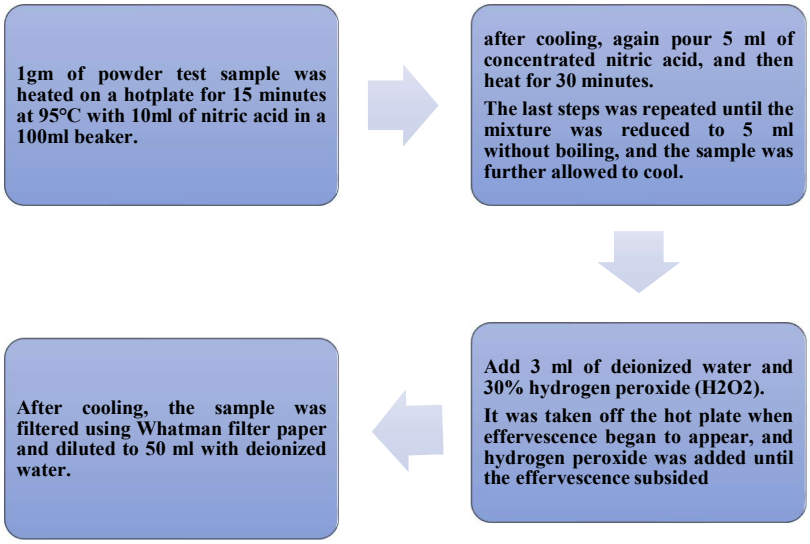
METHODS

Test Sample

The selected polyherbal preparation Kayam Churna (Batch No- #68861) was purchased from the local market, Jhansi, Utter Pradesh, India in December 2021

Heavy Metal Analysis

The selected formulation KC was evaluated for the qualitative and quantitative estimation of metallic elements such as chromium (Cr), lead (Pb), and cadmium (Cd) on the Atomic Absorption Spectrophotometer (Model AA-6880F, Shimadzu) at the Innovation Centre, Bundelkhand University, Jhansi.



Sample preparation method for heavy metal analysis [11]

Phytochemical analysis

The study of preliminary phytochemicals of Kayam churna for evaluation of the presence of plant's secondary metabolites such as Carbohydrates, Amino acids, Steroids, Cardiac glycosides, Saponin, Flavonoids, alkaloids, and Tannins was carried out by **[12, 13]** with some modifications **(Table 1)**.

Cell Culture and Cytotoxicity Assessment

HepG2 cells were obtained from the National Center for Cell Sciences, Pune, India, an ATCC-approved cell repository, and the cells were cultured in Dulbecco's Modified Eagle medium with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics added to the high-glucose medium at 37°C and 5% CO2 in a moist condition. For the cytotoxicity assessment, cells were grown in a 96-well microtitre plate at a density of 10,000 cells/well. Cells were pre-incubated for 24 hours for surface attachment. After 24 hours, Cells were treated with five different concentrations (62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, and 1000 µg/ml) of the test drug for the next 24 hours. At the end of the treatment time duration, the test drug-containing medium was aspirated. To achieve a final concentration of 0.5 mg/ml, 200µl of medium containing 10% MTT reagent was added to each well **[14]**. The mixture was then incubated for 3 hours at 37

°C and 5% CO2 atmosphere. Without damaging the yellow-color formazan crystals, the culture media was entirely removed. The produced formazan was then solubilized by adding 100µl of DMSO solution and gently shaking the plate in a gyratory shaker. A microplate reader was used to measure the absorbance at 570 and 630 nm wavelengths **(Figure 1)**.

Anti-ROS assay

At a density of 3 x 10^5 cells/ml, cells were seeded in a 6-well plate, and they were then incubated in a CO2 incubator at 37°C overnight for 24 hours. Aspirate the used culture medium after 24 hours, then wash the cells with 1ml of 1X PBS. In 2 ml of culture medium, treat the cells with the required concentration of the experimental test formulation (KC), and then incubate them for 24 hours. Once more aspirate the medium, wash the cells with 1 ml 1X PBS, and then re-incubate the cells with 100µM H_2O_2 in 2 ml of culture medium for 2 hours to induce ROS stress. At the end of the experiment, trypsinize the cells into 5 ml FACS tubes and wash them with 1 mL PBS. Centrifuge the tubes at 300 x g for five minutes at 25 °C. Decant the supernatant with caution. Wash twice with PBS. Decant all of the PBS. To prepare a 10µM working solution, dilute the H_2DCFDA stock solution (4mM) with DPBS. Cells were re-suspended in 100µl of H_2DCFDA working solution at a density of 1 x 10^6cells/ml and should be incubated at 37 °C for 30 minutes without exposure to light. Centrifuge the tubes for 5 minutes at 150 x g. In 400µl of pre-warmed DPBS, gently resuspension cells after removing the supernatant. Utilise flow cytometry for analysis, with a 488 nm laser for excitation and detection at 525 nm (FL1) **[15]**.

Data Analysis

For each treatment group, the findings are shown as the mean ± SD of three replicates. The statistical analysis, which was conducted out using Microsoft Excel 2013, used one-way analysis of variance. A P value < 0.05 was considered statistically significant.

RESULTS

Heavy Metal Analysis

Atomic Absorption Spectroscopic (AAS) method was used to identify the presence of heavy metals in our tested formulation (KC). According to our analysis, extremely small concentrations of heavy metals were found in Kayam churna. The concentration of cadmium (Cd) was 0.17ppm, chromium (Cr) was 0.46ppm, and lead (Pb) concentration was not applicable **(Graph1)**.

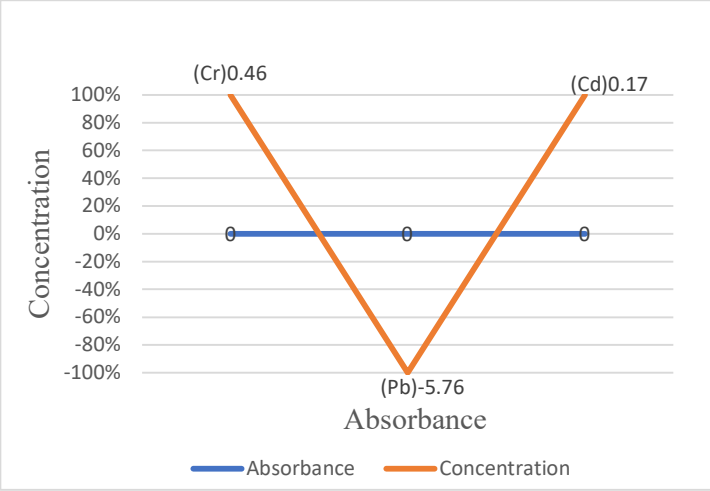
Phytochemical analysis

The phytochemical analysis revealed the presence of secondary metabolites like carbohydrates, steroids, glycosides, saponins, flavonoids, and alkaloids **(Table 1)**.

We used 5 different concentrations of test formulation (KC) to treat cells. There was no evident dose-dependent decline in the viability of the cells, for 24 hours. Cell viability is between 80 to 95%, and treated cells are reduced by 5 to 10% in comparison to untreated cells.

Anti-ROS assay

According to the results of the experiment, cells pretreated with the test formulation KC before being exposed to H_2O_2 showed lower ROS levels than cells that were simply exposed to H_2O_2. This suggests that the tested formulation may have protective effects against the harmful effects of H2O2-induced ROS**.** Thus, the test formulation has anti-ROS activity **(Figure 2) (Table 2)**.



Graph 1: Metal Concentration in KC ppm

Table 1: Preliminary Phytochemical analysis of KC (+ Present, - Absent)

Test	Methanolic Extract	Water Extract
Carbohydrate		
1. Molish's test	+	+
2. Benedict's test	+	-
3. Fehling's test	+	-
Amino Acids		
1. Ninhydrin	-	-
Steroid		
Liebermann- Burchard's test	+	+
Cardiac glycoside test		
1. Keller-killiani	+	+
Saponin glycosides		
1. Foam test	+	+
Flavonoid		
1. Alkaline reagent test	+	-
2. Lead acetate test	+	+
Alkaloid test		
1. Mayer's test	-	-
2. Wagner's test	-	+
Tannin		
1. 5% $FeCl_3$	+	-
2. Lead acetate	+	+
3. Acetic acid	+	+

In-vitro cytotoxicity Assays (MTT)

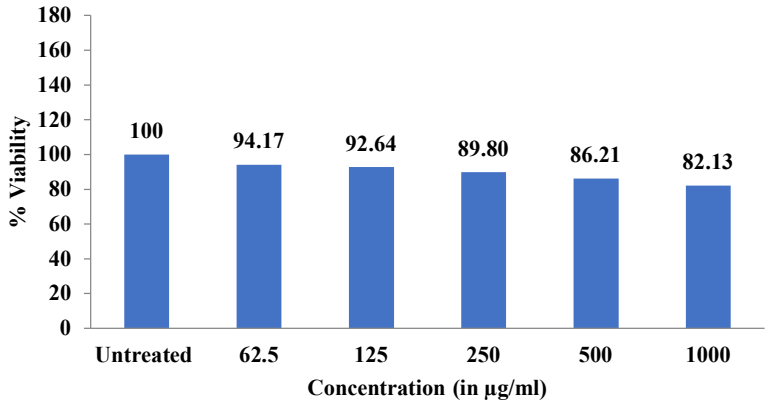


Figure 1: Effect of Kayam churna on HepG2 Cell line

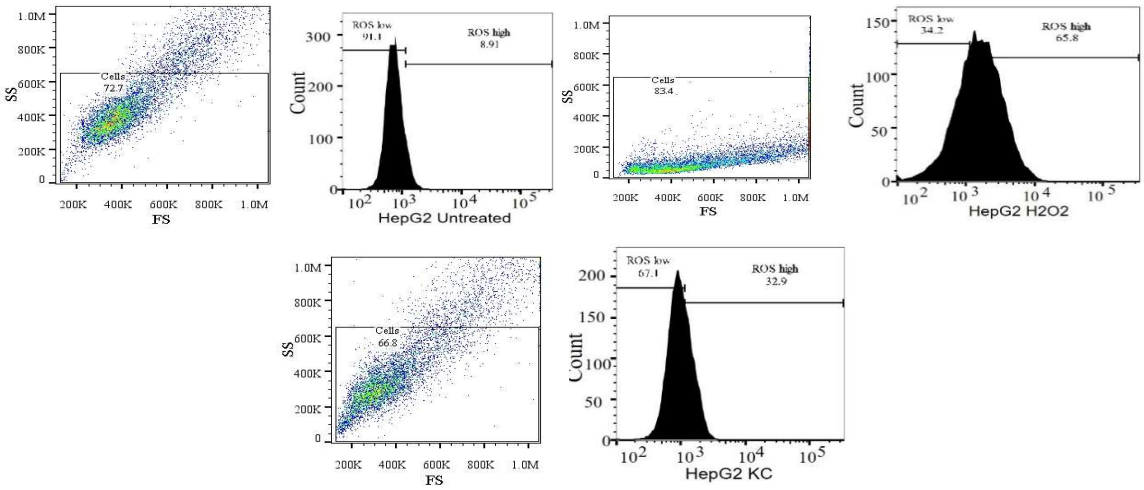


Figure 2: Histogram showing the reducing oxidative stress effect of KC on HepG2 cells compared with positive control (H_2O_2)

Table 2: Frequency of Parent cells (%)

	Untreated Cells	Positive Control	Test Formulation (KC)
ROS high Freq. of Parent	**8.91**	**65.8**	**32.9**
ROS low Freq. of Parent	**91.1**	**34.2**	**67.1**

DISCUSSION

Everybody is skeptical about the quality and safety of Ayurvedic formulations because of the fear of the inclusion of harmful substances, particularly heavy metals. To increase the acceptance of formulations, it is necessary to rule out the appearance of unpleasant elements. Heavy metals may be present in crude medicines due to atmospheric pollution and soil contamination. But minerals and metals are also used in the preparation of Ayurvedic medicines. Heavy metals, on the other hand, have been linked to a variety of negative

effects **[16]**, including status epilepticus, fatal neonatal encephalopathy, hepatotoxicity, congenital paralysis and deafness, and developmental delay. Many case studies have revealed serious adverse effects caused by heavy metals in Ayurvedic and other herbal medicines **[17]**. Lead and cadmium are reportedly quite concerning because they have a negative impact on people's health **[18]**. The permitted limits for heavy metals in Ayurvedic medicines are 10 ppm for lead, 1 ppm for chromium, and 0.3 ppm for cadmium **[19]**. Our heavy metals analysis results revealed that the concentrations of heavy metals like lead, Cadmium, and Chromium are very low, indicating that the selected Ayurvedic formulations are safe from metal toxicity. However, the level of these heavy metals may also differ from batch to batch because metals are typically transferred from the environment, water, and soil to the plant materials. However, the tested medicine batch was found to be safe in terms of metal toxicity.

Phytochemical evaluation of tested formulation KC can reveal the wealth of chemicals found in them. Secondary metabolites generated from plants are a valuable source of novel medications, and some are found as drug precursors **[20]**. Flavonoids are low molecular weight secondary metabolites having anti-aging, antimicrobial, and antioxidant effects. Because of their capacity to block carcinogenesis at various stages, they have the potential to be effective cancer chemotherapeutic agents **[21]**. Tannins are water-soluble polyphenols with antibacterial characteristics that can extend the shelf life of preparations **[22]**. The phytochemical evaluation revealed that Kayam churna contained secondary metabolites such as alkaloids, flavonoids, tannins, sterols, and glycosides, which together conferred the formulation's pharmacological potential. The cytotoxicity study suggests that there is no significant reduction in cell viability at any concentration of selected Ayurvedic preparation. Flow cytometry investigation of ROS levels in HepG2 cells using an H_2DCFDA-based assay demonstrated that H_2O_2 caused oxidative stress in HepG2 cells by producing high levels of ROS generation. ROS are a byproduct of regular cellular respiration and are primarily involved in cellular damage and apoptosis **[23]**. Cells treated with 100µM H_2O_2 resulted in excessive generation of ROS as compared to untreated cells. After the treatment of selected Ayurvedic preparation, damaged cells showed a reduction in ROS generation. This finding suggests that KC may protect HepG2 cells in a ROS-independent manner.

CONCLUSIONS

In our study, KC did not have a heavy metal level beyond the permissible limit and did not adversely impact cell viability even at

the highest concentration (1000ug/ml). The presence of very low concentration (BDL) of the tested heavy metals, namely lead (Pb), Copper (Cu) and Cadmium (Cd) suggested that Ayurvedic preparation has been developed according to established Ayurvedic standard protocols. The presence of phytochemicals confirmed its therapeutic potential and also the anti-ROS study revealed that Kayam churna has the potential to reduce intracellular ROS generation and reduced oxidative stress. As a result, KC was shown to be a safe agent for the aforementioned applications. In addition, we provide high-quality supporting data that may increase their market value. However, the extensive study may yield additional data on this.

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