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Original Article

Pharmacology Section

Biochemical Estimation in an Acute Toxicity Study of Narayana Chenduram- A Siddha Formulation

PM SUKALA[1], R MUTHUSAMY[2], V TAMILALAGAN[3], S BASKARAN[4], ARBIND KUMAR CHOUDHARY[5]

ABSTRACT

Introduction: Chendurams are more potent and its potency is further increased when combined with herbal juices. Chenduram is known to be effective when it is given at low concentration. Narayana Chenduram (NC) is a metal based siddha formulation that contains heavy metals like mercury, cinnabar, arsenic along with sulphur/sulphides. NC is used to treat Parkinson's disease along with the polyherbal formulation Athimathura Gritham (AG).

Aim: To evaluate the health status of the animals under acute oral toxicity study of NC. Biochemical estimation of heavy metals in liver, kidney, brain and serum was carried out. Histopathological study was also performed in liver and kidney.

Materials and Methods: The present experimental study was conducted in Department of Anatomy, University of Madras, Chennai, India from October 2009 to December 2009. The heavy metals present in NC was analysed by Inductively Coupled Plasma Optical Emission Spectrophotometry (ICP-OES) using acid digestion method. A single oral dose acute toxicity study of NC was conducted using acute toxic class method as per Organisation for Economic Cooperation and Development (OECD) Guidelines for the testing of chemicals. It was done using limit test method. The study was conducted by giving NC at a single dose of 2000 mg/kg body weight mixed in 10 mL of honey/kg body weight and rats were observed for 14 days for toxic signs. Total five animals were tested under this method to determine Lethal Dose 50 (LD50). On day 15, the distribution of heavy metals in liver, kidney, brain and serum was determined by ICP-OES using acid digestion method and was compared with control. Food and water intake, body weight were recorded before and after drug administration as per the guidelines. Histopathological examination of liver and kidney was performed in the same animals and compared with control.

To find the effect of given adjuvant (honey) under acute oral toxicity study, NC at a single dose of 1000 mg mixed with 10 mL of honey/kg body weight was administered to one group of animals and NC at a single dose of 1000 mg mixed with 10 mL of sesame oil/kg body weight was given orally to another group of animals. After administration of test drug, the rats were observed for 14 days for toxic signs and on 15th day they were sacrificed to study histological changes in kidney and liver among these two groups. Adjuvant treated control group of animals were administered only with 10 mL of honey at a single dose and observed for 14 days and sacrificed on day 15 to study histology of liver and kidney and compared with control.

Results: No mortality was observed at a single dose of 2000 mg/kg under acute toxicity study. Hence, LD50 was greater than 2000 mg/kg body weight. ICP-OES analysis showed that the mean concentration of mercury was five times more than that of mean concentration of arsenic in the given sample weight of NC. Under acute toxicity study, after oral administration of NC at a single dose of 2000 mg/kg body weight, serum showed more significant accumulation of mercury than arsenic when compared to control groups. A single dose of 2000 mg NC produced hepato-toxicity and renal toxicity. At a single dose of NC at 1000 mg mixed with 10 mL honey showed less histopathological changes when compared with NC mixed with sesame oil. Adjuvant (honey) treated group did not show any histopathological toxicity in liver and kidney when compared with control.

Conclusion: Though there was no mortality at a single dose of 2000 mg/kg body weight, serum showed marked accumulation of mercury that indicates toxicity. It produced signs of histopathological toxicity in liver and kidney. Dose dependent change was observed. Hence, it is recommended to use Chenduram at low doses.

Keywords: Cinnabar, Histopathological examination, Parkinsonism, Siddha medicines, Sulphur

INTRODUCTION

Metals like gold, lead, copper, iron and zinc are used to prepare the siddha drugs based on ancient practices of Panchabuthas. Siddha medicine are utilised as alternative medicine for treatment of various diseases due to its efficacy, more availability, less expensive and are generally believed to be safe. The process of ageing brings along with it a numerous degenerative physical and mental changes which are managed in Siddha system by the rejuvenating practices involving simple herbal formulations (powerful antioxidants) prescribed in specific dose to be taken for a particular length of period and techniques. In Siddha system of medicine, metallic herbal preparations are given as chendurams, bhasmas, parpam and so forth. They are prepared by repeated incinerations of metals like mercury, gold, silver, arsenic, copper, or their salts (preferably sulphides) are processed with indigenous herbal juices to detoxify it [1]. Chendurams are more potent and its potency is further increased when combined with herbal juices. Chenduram is known to be effective when it is given at low concentration [2]. NC is a mineral/metallic sulphide formulation containing mercury, arsenic, cinnabar with sulphur. In Western medicine, mercury is used as a cure for syphilis, mercury chloride are used as diuretics and mercury amalgam is still used for filling teeth. Arsenic compounds have also been used in the treatment of syphilis, amoebic dysentery and trypanosomaiasis (Arsenic trioxide has been used for the induction of apoptosis in leukaemia cells). NC has been prepared by process of sublimation as per siddha literature [3,4]. By the process of sublimation, their size is reduced from crystalline to nanoform like Linga Chenduram, Poorna Chandrodaya Chenduram [5].

Chenduram is in fine powder form and it has been taken along with the honey or ghee as an adjuvant, which enhances bioabsorption of the medicine. While mixing with honey, it is presumed that the atomised form (free atoms) saves life from death and decay [6].

NC along with the polyherbal drug AG is used as a combined therapy to treat neurodegenerative disease like Parkinsonism. AG is a polyherbal drug processed in the freshly prepared cow's ghee as described in siddha literature [7]. It contains the following ingredients like *Elettaria cardamomum* Maton, *Alternanthera sessilis, Cuminum cyminum* Linn, *Costus speciosus, Coriandrum sativum* Linn, *Cinnamomum verum Presyl, Ludwigia octovalvis, Hygrophila auriculata, Hemidesmus indicus, Vitis vinifera, Santalum album* Linn, *Vetiveria zizanioides, Zingiber officinale* Rose. Many parts (roots, stem, leaves, fruit, seeds and rhizomes) of these plants have been individually reported to exhibit pharmacological actions such as diuretic, hepatoprotective, renoprotective, anti-inflammatory, antioxidant, anthelmintic, nervine tonic, cardiac and liver tonic and analgesic [8-18].

Both the drugs were received as a sample from a Siddha Physician, Government Anna Hospital, Chennai, Tamil Nadu, India. Because of limited availability of the data on the safety/toxic profile of the metal based drug NC, the present study was undertaken. ICP-OES was employed to analyse the heavy metals present in NC, which was reported in the previous study [19]. The distribution of heavy metals in liver, kidney and serum of Wistar albino rats were analysed by ICP-OES at a single dose of test drug at 2000 mg/kg body weight under acute oral toxicity study. Since NC is used to treat Parkinson's disease, whole brain was also analysed for the presence of heavy metals. To evaluate the health status of the animals under acute oral toxicity study of NC at 2000 mg/kg body weight, histological study of liver and kidney was carried out and compared with control. The biochemical parameters [19], haematological investigations were reported [20] for the same group in authors previous work. Here, the microscopic examination of liver and kidney was done in all groups and compared with the control. Due to large number of parameters studied under this project, different aspects have been presented in different papers to keep focus and interest for the readers.

MATERIALS AND METHODS

This animal experimental study was carried out and the project was approved by IAEC (Institutional Animal Ethical Committee) University of Madras, Chennai, Tamil Nadu, India and Committee for Purpose of Control and Supervision of Experiments on Animals (CPSEA), and was conducted in accordance with the standard procedures of IAEC. The project approval number was IAEC No.01/017/04. It was conducted in Department of Anatomy, University of Madras, Chennai, Tamil Nadu, India from October to December 2009. Healthy adult male Wistar albino rats weighing about 200 to 230 gm of body weight were included in this study. Rats were housed in pairs and maintained under standard atmospheric condition of 12 hours light/12 hours dark cycle at 21°C to 26°C and 30 to 60% humidity. Animals were fed with standard rat pellet diet (Hindustan lever limited, Mumbai, India) and water ad libitum.

Rats were divided into five groups: Six animals in each group except in Group II- five animals. **Group I-**normal control (received distilled water orally of 2 mL/kg body weight, **Group II-**Lethal Dose (LD) of NC once at a dose of 2000 mg mixed in 10 mL of honey/kg body weight as an adjuvant, **Group III-**NC once at a dose of 1000 mg mixed in 10 mL of honey/kg body weight as an adjuvant, **Group IV**-NC once at a dose of 1000 mg mixed in10 mL of sesame oil/kg body weight as an adjuvant, **Group V-**Adjuvant treated control (Honey-Dabur Co.) 10 mL/kg body weight.

Determination of heavy metals: The heavy metals present in NC were analysed by ICP-OES using acid digestion method [21].

Acute Oral Toxicity Study of Narayana Chenduram (NC)

An acute oral toxicity study of NC was conducted using acute toxic class method as per OECD guidelines 425 [22]. It was done as per limit test method. Group II animals were deprived of food overnight but water was provided ad libitum. In this method, NC at a single dose of 2000 mg/kg body weight mixed in 10 mL of honey/kg body weight was given orally to one animal and was observed for mortality, general appearance of skin, respiration, locomotor activities, tremors, sleep and coma for 24 hours after administration and observed daily for 14 days for any toxic signs. These observations were recorded by videograph. The animal survived, hence additional four rats were administered orally with the test dose. Total five animals were tested under this method to determine LD50.

On day 15, animals were sacrificed by intraperitoneal administration of overdosage of sodium pentobarbital at the dose of 80 mg/kg bodyweight. Approximately, 3 mL of blood was collected through cardiac puncture for metal analysis as well as for biochemical [19] and haematological investigations [20]. Rats were then transcardially perfused with 10% normal saline followed by 10% formal saline. Kidneys and liver were removed immediately for metal analysis and also for histological study and compared with control. Brain was removed immediately from the same group of animals and utilised for metal analysis by ICP-OES using acid digestion method and compared with control. Food and water intake, body weight were recorded before and after drug administration as per OECD guidelines.

Under acute toxicity study, to find the effect of given adjuvant (honey), a single dose of 1000 mg of NC mixed with 10 mL of honey/kg body weight was administered to Group III animals and NC at a single dose of 1000 mg mixed with 10 mL of sesame oil/kg body weight was given orally to group IV animals. After administration of NC, the rats were observed for 14 days for toxic signs and on 15th day it was sacrificed to study histological changes in kidney and liver among these two groups. Adjuvant treated control group V animals were administered only with 10 mL of honey at a single dose and observed for 14 days and sacrificed on day 15 to study histology of liver and kidney and compared with control. The food, water consumption and body weight were recorded.

Determination of Heavy Metals in Plasma, Liver, Kidney and Brain by ICP-OES using Acid Digestion Technique

Procedure for plasma: Plasma was separated by centrifugation at 3000 rpm for 15 minutes and stored on ice until required. Two mL of a mixture of nitric, perchloric and sulphuric acid was added (6/1/1 by volume) to the plasma. Digest and evaporate at 250°C to near dryness. Cool to room temperature, dissolve the residue in 6 mL of distilled water and 5 drops of concentrated Hydrochloric Acid (HCL) and heat again for 5 minutes. Cool the solution to room temperature. Add 0.3 mL of ammonium pyrroline dithiocarbamate and one drop of m-cresol purple indicator (1g/L) to the liquid, shake the mixture and adjust to pH 9 with ammonia water. Add 1 mL of methyl isobutyl ketone to the mixture and shake it again for 2 minutes. Separate the two phases by centrifuging at 750 rpm for 2 min. Further aspirate 0.5 mL of the thin organic layer and transfer into 5 mL of pyrex test tubes standing in a rack in front of the graphite tube. Start the automatic HGA-76 sequence program by which 20 µL of the mixture is aspirated and injected into the graphite tube.

Procedure for Liver, Kidney and Brain: Remove liver, kidney and brain. Dry these tissues in hot air oven at 80°C. Then powder it in a mortar with its pestle separately and take 1 gm of powder and add 10 mL of 10% of nitric acid and allow it to boil for 30 minutes and add 2 mL of hydrogen peroxide. Again, boil for 30 minutes. Make-up the residue upto 25 mL with the deionised water. Filter it with the Whatman No.1 filter paper and analyse it.

RESULTS

In the acute toxicity study, group II animals after five hours of administration of NC at 2000 mg/kg showed restlessness. No other signs of toxicity and mortality were observed upto 14 days in group II. Therefore, LD50 is greater than 2000 mg/kg. It did not significantly affect food, water intake and body weights of the treated rats when compared with control. No gross morphological changes in liver and kidney were observed. In group III, IV and V, animals showed no signs of toxicity and mortality upto 14 days of observation. No changes were observed in food and water intake and also body weights of these groups when compared with control.

Analysis of metals by OPTIMA 5300DV ICP-OES [Table/Fig-1] shows the mean concentration of mercury (258.75 mg/L) was more than five times that of mean concentration of arsenic (44.92 mg/L) in the sample weight (0.02145 g/25 mL) of NC. Sulphur was detected at a mean concentration of 55.67 mg/L. The mean levels of mercury and arsenic were raised in NC as compared with WHO recommended level of 0.001mg/L and 0.01mg/L respectively [23].

Analyte name	Concentration of sample ppm or mg/L
Cr 267.716	<0.24
Pb 220.353	<1.43
Hg 253.652	258.75
As 188.979	44.92
S 181.975	55.67

[Table/Fig-1]: Determination of metals in Narayana Chenduram (NC). Sample weight=0.02145 g/25 mL; Cr: Chromium; Pb: Lead; Hg: Mercury; As: Arsenic; S: Sulphur; Above element value is given along with spectrophotometry set point resolution i.e. denoted in numbers

The analysis showed that metals in NC were existing in sulphide form. Under acute toxicity study, after oral administration of NC at a single dose of 2000 mg/kg body weight, [Table/Fig-2] shows various elements and mean concentration which were detected in various organs. On day 15, the liver exhibited the highest uptake of arsenic at a mean concentration of 0.357 mg/L followed by kidney (0.188 mg/L), brain (0.118 mg/L) and serum (0.096 mg/L). No mercury was found in liver, kidney and brain but in serum the mercury was found to be in mean concentration of 4.754 mg/L than arsenic (0.096 mg/L). The serum of group II animals showed that the accumulation of mercury was higher than four times than that of Arsenic. In Group I, animals showed that Hg, As and S were below detectable limit in liver, kidney, brain and serum.

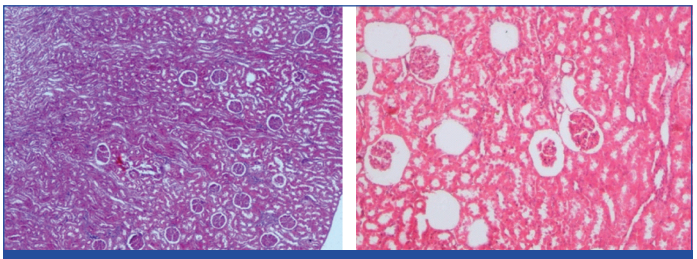
Sample	Analyte name	Mean concentration (mg/L)
Liver	As	0.357
	Hg	BDL
	S	207.3
Kidney	As	0.188
	Hg	BDL
	S	97.24
Brain	As	0.118
	Hg	BDL
	S	79.01
Serum	As	0.096
	Hg	4.754
	S	337.0

[Table/Fig-2]: Analysis of Narayana Chenduram (NC) in tissues of Group II animals. BDL: Below detectable limit

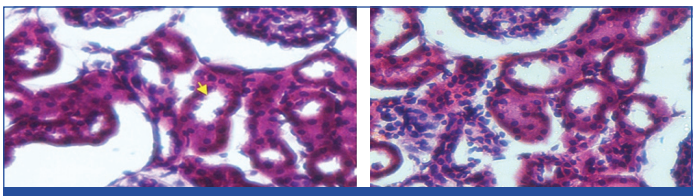
Histopathological Changes

Effect of NC in kidney and liver

In control group I [Table/Fig-3], the microscopic examination of the kidney revealed normal architecture. In the acute toxicity study, animals treated with NC at 2000 mg/kg body weight in group II [Table/Fig-4], lack of regular contour of glomeruli and extensive loss of lining epithelium of the tubules were noted. These features were predominantly subcapsular, widening of filtering space, and mononuclear cell infiltration. Under acute toxicity study, NC treated group III at 1000 mg/kg body weight, most of the Proximal Convoluted Tubule (PCT) around the glomerulus in the cortex showed narrow lumen with brush border in honey treated rats [Table/Fig-5]; whereas in oil treated rats, most of the PCT around the glomerulus in the cortex showed wider lumen with loss of brush border [Table/Fig-6]. The mononuclear cell infiltrations were present in both cases. Dose dependent results were observed and higher dose show marked changes. Adjuvant treated group V did not show any marked changes in kidney when compared with control group.

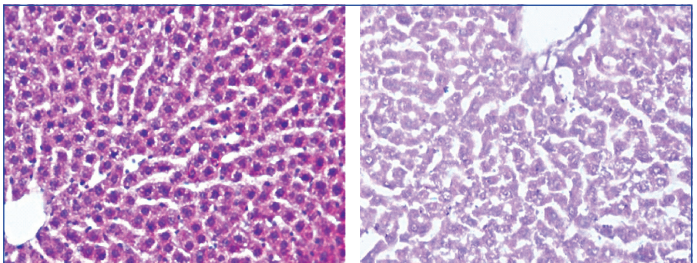


[Table/Fig-3]: Histology of control kidney. H&E stain under 4X showing normal architecture. **[Table/Fig-4]:** Histopathological changes in kidney with 2000 mg of Narayana Chenduram. H&E stain-10X showing lack of regular contour of glomeruli, widening of filtering space and mononuclear cell infiltration. (Images from left to right)

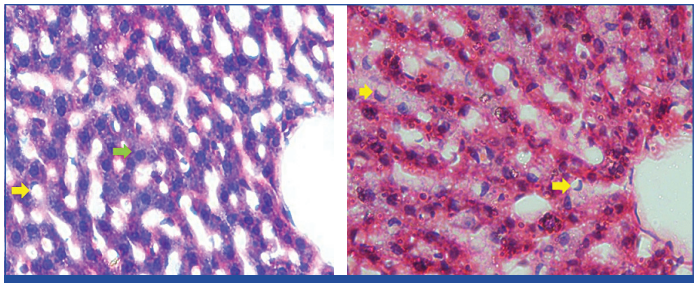


[Table/Fig-5]: Histopathological changes of kidney with 1000mg of NC mixed with honey. H&E stain – 40X showing PCT around glomerulus with narrow lumen showing brush border (yellow arrow). **[Table/Fig-6]:** Histopathological changes of kidney with 1000 mg of NC mixed with sesame oil. H&E stain under 40X showing PCT around glomerulus showing wider lumen with loss of brush border. (Images from left to right)

Histopathological examinations of liver of control animals [Table/Fig-7] showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and a central vein. The liver of the animals given with NC at 2000 mg/kg body weight, showed necrosis with inflammatory cells, central venous congestion, parenchyma disorganisation and distended perisinusoidal space [Table/Fig-8]. Under acute toxicity study, animals treated with NC at 1000 mg/kg bodyweight mixed with honey. [Table/Fig-9] shows few degenerating hepatocytes with crescentic nucleus toward periphery and more distended hepatocytes with centrally placed large nucleus, whereas the animals treated with NC at 1000 mg/kg body weight mixed with sesame oil showed shrunken hepatocytes and more number of degenerating hepatocytes with crescentic nucleus towards periphery [Table/Fig-10]. In group V the adjuvant treated rats did not show any significant changes in liver when compared with control group.



[Table/Fig-7]: Histology showing liver of control rat with normal architecture. H&E stain 40X. **[Table/Fig-8]:** Histopathological changes of liver with 2000 mg of NC. H&E stain under 40X showing disorganisation of parenchyma, degeneration of hepatocytes and distended perisinusoidal space. (Images from left to right)



[Table/Fig-9]: Histopathological changes of liver with 1000 mg of NC mixed with honey. H&E stain under 40X showing few degenerating hepatocytes (yellow arrow) with crescentic nucleus at the periphery, more distended hepatocytes with centrally placed large nucleus. (green arrow). **[Table/Fig-10]:** Histopathological changes of liver treated with 1000 mg NC mixed in sesame oil. H&E stain. 40X-showing most of the hepatocytes undergoing degeneration with crescentic nucleus towards periphery (yellow arrow). (Images from left to right)

DISCUSSION

Mercury and Arsenic are in higher amounts in NC than WHO recommended level in food and drug, respectively [24]. Mercury is the main component in NC. It is used as a catalytic agent and used in combination with sulphur to control the fluidity of mercury. Twenty five parts of mercury by weight could only take up four parts of sulphur [6,25]. The excess sulphur volatilises forming HgS. Even though the Hg and As are present at higher level in NC than regulatory limits, they are existing in sulphide form which is highly insoluble, less toxic and less absorbed than other forms of mercury. Under acute toxicity study, on 15th day, liver sample showed a low accumulation of As in the liver, kidney, brain and serum when treated with maximum dosage (2000 mg/kg) of NC. Traditional Chinese Medicine containing As as realgar when treated with higher dose (600 mg/kg) also showed low levels of As in liver and kidney tissues of mice when compared with sodium arsenite (36 mg/kg) and arsenate (88 mg/kg) [26].

It was found that cinnabar ore contains approximately 98% arsenic, could contribute to increase As level in tissues. The high levels of As and Hg from Traditional Chinese Medicine (TCM) were excreted via faeces and only small amount in urine. It is possible due to low solubility of realgar (As_4S_4) or orpiment (As_2S_3) and Hg as cinnabar present in the TCM. It is poorly absorbed from gastrointestinal tract, accumulate in tissues and readily excrete via faeces [27]. In hamsters, orpiment is poorly absorbed and over 82% is found in faeces within three days after oral dose when compared to more soluble sodium arsenate, in which only 12% is excreted in faeces [28]. Rats exposed to HgS at a higher oral dose (2.5 g/kg body weight) has shown accumulation of mercury in liver and caused neurotoxic effect on vestibule-ocular system in guinea pigs [29]. The oral administration of HgS (1.0 g/kg/day) for consecutive 5 days treatment had no significant effect on body weight and no peripheral neurotoxicity in rats. After consecutive 14 days of oral HgS (1.0 g/kg/day), the Hg level of the blood reached to about 0.5 ppm, which started to produce peripheral neurotoxic [30]. After 14 days cessation of administration, complete recovery from peripheral neurotoxicity was concomitant with lowering of Hg level of blood to about 0.1 ppm as compared to control. It is suggested that the blood Hg level can be a good indicator for peripheral toxicity induced by mercurial compounds.

Mercury is used as a nervine tonic and for restoring normalcy to collapsing patients. Mercury acts as a rejuvenator and facilitates the learning process at small doses of 15 mg/kg [31]. Arsenic preparations show no acute toxicity and have an analgesic activity [32]. It has been suggested that, it can cure all diseases if it is properly prepared and used. Linga Chenduram (LC) at 100 mg/kg body weight of rats revealed antipyretic activity against brevers yeast induced pyrexia and analgesic activity against hot plate induced analgesia but at 200 mg/kg body weight of albino rats, LC did not produced significant effects in these activities [33].

Histopathological study showed NC with honey treated group showed comparatively less damage in liver and kidney than NC with oil treated group. In acute and subchronic toxicity studies of NC in wistar rats, the biochemical and haematological parameters showed systemic toxicity [19,20]. An acute toxicity study of Gowri Chinthamani Chenduram (GCC), upto 640 mg/kg body weight did not show adverse effects and mortality in wistar albino rats but chronic toxicity of GCC at 160 mg/100 gm body weight for 90 days caused renal and hepatic changes [34].

In acute toxicity study the adjuvant (honey) treated group showed no toxicity. In subchronic study, the biochemical and haematological parameters of honey treated group showed less significant changes compared to control group [19,20]. This might be due to contamination and long term usage of it. Honey contamination with excessive trace elements have been reported previously [35]. The average daily consumption of honey recommended is 0.8 g/kg/day [36]. The usage of contaminated honey above recommended level overtime, might have produced toxic effect. It shows that for clinical use, selection of honey with high levels of antibacterial, antioxidant, anti-mutagenic, antiinflammatory activities are important to maximise therapeutic effect.

The animals treated with NC+AG at therapeutic dose level showed no significant changes in haematological and biochemical parameters [19,20]. In Rotenone induced Parkinsonian rat model, when NC+AG given at therapeutic dose level, it was found to be effective and showed no toxic effect to vital organs especially kidney and liver [unpublished data]. One of the study showed that their acute study of Kalamega Narayana Chenduram (KNC) at 2000 mg/kg in Wistar albino rats did not show mortality. Twenty eight days repeated oral toxicity studies of KNC did not exhibited any toxicity in haematological, biochemical parameters and also histological changes in liver and kidney [37]. Nanoform particles of chenduram can carry peptide drugs or diagnostic agents complexed by adsorption or covalent attachment, may improve the transport properties and stability of transported agent [38]. Once the nanoparticle reaches the desired target, release can be achieved by one or more mechanism such as desorption, diffusion and nanoparticle erosion [39]. The anti-inflammatory, antioxidant and antimutagenic activities of honey and also activities of phytoconstituents present in AG may interact with the sulphide form of heavy metals in NC at the molecular level and may change its chemical behaviour. The extract of fenugreek is capable to change the chemical behaviour of nickel by molecular interaction between nickel and phytoconstituents in fenugreek [40]. These effects suggest that methods of correct preparation, duration of drug administration and drug combination, age and appropriate dosage may greatly influence toxicity of sulphide compounds.

Limitation(s)

The major limitation of the study was that, if advanced technique like fourier transform infrared spectroscopy is applied for quantitative/biochemical estimation of NC to identify its purity, presence of different trace elements, as well as functional groups like alcohols, phenols, aldehydes, ketones, nitro compounds etc., which are used for treating various life-threatening diseases, the exact mechanism of action of NC alone could be explained at the therapeutic dose level. It might have made present study results more robust without impairing the interpretation of the data.

CONCLUSION(S)

It can be concluded from the study that higher doses can produce signs of histopathological toxicity in liver and kidney. Authors also studied in rotenone induced parkinsonian rat model, NC+AG given at therapeutic dose level, it was found to be effective and showed no toxic effect to vital organs especially in liver and kidney. Chendurams are effective only in low concentration and when combined with polyherbal drug it is found to be more effective. Authors strongly recommend the need of preclinical and clinical evaluation of the metallic herbal formulations for their wide spread acceptance among scientific community.

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PARTICULARS OF CONTRIBUTORS:
1. Associate Professor, Department of Anatomy, Government Erode Medical College, Perundurai, Erode, Tamil Nadu, India.
2. Retired Professor, Department of Anatomy, Dr A.L.M Post Graduate Institute of Basic Medical Sciences, Chennai, Tamil Nadu, India.
3. Professor, Department of Anatomy, National Institute of Siddha, Chennai, Tamil Nadu, India.
4. Professor, Department of Biochemistry, Indira Gandhi Medical College and Research Institute, Puducherry, India.
5. Assistant Professor, Department of Pharmacology, Government Erode Medical College, Perundurai, Erode, Tamil Nadu, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Arbind Kumar Choudhary,
Department of Pharmacology, Government Erode Medical College, Perundurai,
Erode-638053, Tamil Nadu, India.
E-mail: arbindkch@gmail.com

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