Evaluation of Herbal Formulation for Diuretic Activity in Experimental Animals.

Submitted in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy

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ABSTRACT

Objective: The present study was designed to evaluate the diuretic activity of herbal formulation (Gokhru Kadha). To investigate/propose probable mechanism of action of herbal formulation.

Method: Animals were divided into 5 groups of 6 Swiss mice each. Group 1 served as control and administered with vehicle. **Groups II, III and IV*:*** Served as treatment group and a single dose of 20 mg, 40 mg and 60 mg/kg of HF administered orally to group II, III and IV respectively. **Group V:** Served as standard, treated with furosemide (10 mg/kg), and dissolved in 0.9% normal saline with CMC.

Two days prior to experiments, mice were kept in metabolic cages with free access to food and water for acclimatization. Four hours before testing, the animals were fasted, with free access to drinking water only. All animals were given an oral loading of normal saline (5% by wt.).

For Acute- Freshly prepared doses of test and standard drug were administered to test and standard animal group respectively. Immediately after dosing, animals were kept in metabolic cages for 6 hours and finally urine was collected, measured and filtered at the end of 6 hours [8] for various biochemical estimations.

For Chronic- Daily doses of test and standard drugs were given to 4-hour fasted test and standard drug animal groups respectively for 8 days. Urine volume, urinary electrolyte level will be estimated on 24 hrs. collected urine. On 8th day, additionally urinary glucose will be estimated.

Result: The Polyherbal formulations produced significant increase in Na+, K+, Cl- excretion, caused alkalinization of urine, showed strong Diuretic index, saluretic index and Natriuretic index. The higher dose of HF produced comparable effect to that of furosemide.

Conclusion:

As evidenced by the outcome of this study, it is reasonable to infer that the gokhru Kadha possess a significant diuretic activity in mice. Further in-depth studies are required to assess the diuretic activity in combination with the synthetic drugs and to elucidate possible mechanism of action.

KEYWORDS

I. Diuretics

II. Furosemide

III. Acclimatization

Approval for Bachelor of Pharmacy

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Declaration

I declare that this written submission represents my ideas in my own words and where others ideas or words have been included, I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that any violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken.

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TABLE OF CONTENT

INDEX

LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOGRAPHS

KEYWORDS AND GLOSSARY

I. Diuretics-
II. Furosemide
III. Tribulus terrestris
IV. Male Swiss mice
V. Acclimatization
VI. Acute diuretic study:
VII. Chronic diuretic activity:
VIII. Herbal formulation
IX. Urine electrolytes

1.INTRODUCTION

Diuretics play very important role in increasing the rate of urine flow, sodium excretion and to maintain the volume and composition of body fluids in a various clinical Disorders.[1]

They are used in treatment of fluid retention conditions such as cardiac failure, cirrhosis, and nephritic syndrome that lead to fluid overload in the body. Diuretics are used as part of therapeutic approach to control fluid overload manifesting as ankle swelling, ascites, and/or pulmonary oedema, help balance fluid, and help shift fluid out of the interstitium, leading to significant symptomatic relief and improved health-related quality of life in patients.[2]

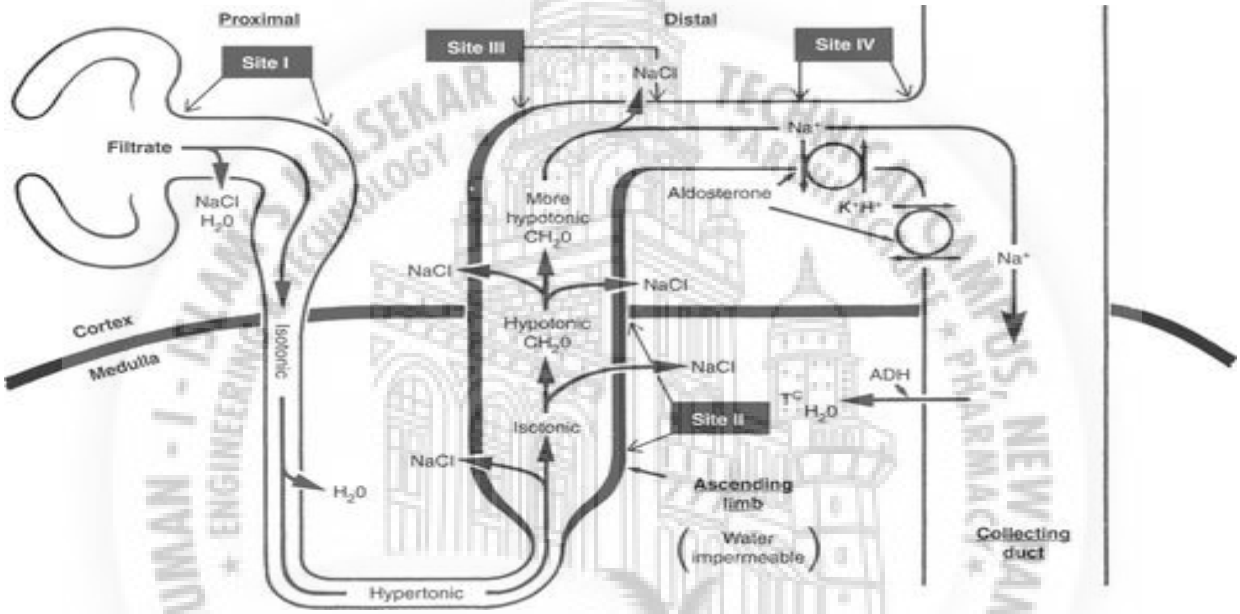

Fig.1.1 Mechanism of Diuretics

Naturally occurring diuretics inhibit Na^+ reabsorption and inhibit secretion of ADH but have the adverse reactions including impotence, fatigue, weakness etc. Few example of Natural Diuretics include caffeine, alcohol and wine. [1]

Several adverse effects such as electrolyte abnormalities (hypokalaemia, hyperuricemia, and hyponatremia), acid–base imbalance, metabolic abnormalities (hyperglycaemia and hyperlipidaemia), and acute hypovolemia are associated with major diuretics, such as the loop and thiazides diuretics. [3]

It is, therefore, important to search for a diuretic that is comparatively free from such untoward and sometimes lethal adverse effects.

In Ayurvedic system several plants such as Crataeva nurvala, Dolichos biflorus, Tribulus terrestris, Dendrophthoe falcata, Boerhaavia diffusa, Saccharum officinarum, Butea frondosa, Boerhaavia repens, Boerhaavia rependa, Homonia riparia, Centratherum anthelmintivum, Vitis venifera and Duranta repens have shown and claimed excellent diuretic activity. [1]

About 88% of the world's inhabitants rely mainly on traditional medicine for their primary health care. Scientific validation of reported pharmacological activities of herbal formulations is essential in order to justify the acceptability of herbal formulations in modern system of medicine· Evaluation of herbal preparation is a fundamental requirement of industry and other organizations dealing with ayurvedic and herbal products[4,5]

Gokhru Kadha is an ayurvedic formulation consisting of *Tribulus terrestris* and *woodfordia fruticosa, Acacia Nilotica* manufactured by Sandu Pharmaceutical Pvt. Ltd.

It is used for several renal disorders such as Urinary calculus, urinary tract infection, oliguria, haematuria, glomerulonephritis, reduces pain and anti-inflammatory. But the scientific reports on the use of the selected formulation as diuretics is lacking in literature upto the present time.

Thus, to evaluate the diuretic activity of this Herbal formulation in swiss albino mice was the aim of the present study. The study will also support its use as diuretics and if it can be used in combination with the other allopathic drugs to decrease the dose and to have synergistic activity.

2. LITERATURE REVIEW

2.1 Ayurvedic Herbal Formulation-GokhruKadha

- SanduGokhruKadha is a herbal Ayurvedic medicine. It is decoction of Gokhru or Tribulus and chiefly indicated in urinary disorders.
- GokhruKadha has diuretic activity. It increases passing of urine and helps in dissolution of a calculus. It reduces swelling in the body. It helps to reduce the size of stones and this helps in expulsion of stones from the body. The Kadha gives relief from symptoms such as painful urination-burning and itching sensation, in conditions where blood is found in urine. It has also been found very important to relieve pain associated with Urinary calculi and avert the reoccurrence of stones if used routinely.

Figure 1: Marketed GokhruKadha

- **Manufacturer**: Sandu Pharmaceuticals
- **Availability**: Online and at medical stores
- **Type of medicine**: Ayurvedic Medicine
- **Main Indication**: Urinary disorders, Semen disorders

2.2 Ingredients of SanduGokhruKadha

Each 5 ml contains: -

- Gokhru
- Dhatiful
- Babool

2.3 Gokhru is used in Ayurveda in the form of powder or decoction for the treatment of:–

- Urinary Disorders (painful micturition, calculus affections, urinary discharges)
- Stones
- Impotency
- Cough-Asthma
- Gonorrhoeal rheumatism
- Dysuria

2.4 Benefits of SanduGokhruKadha

- It **stimulates** production of urine.
- It helps to **dissolve calculus** in the urinary tract.
- It reduces **inflammation** or swelling.
- It gives relief in **pain** that is related to burning and itching sensation during urination.
- It **relaxes** the **urinary tract membranes**.
- It **rejuvenates** the reproductive system.

Its **saponins** and flavonoid content act as **hormonal precursors.**

2.5 Literature Survey on Pharmacological activities of phytochemicals present in gokhruKadha.

Table: 2.1: - Pharmacological activities of phytochemicals present in gokhruKadha.

Sr. No	Phytochemical	Reported Activity	Reference
1.	**TribulusTerrestris [Gokhru]**	Antiurolithiatic	6
		Diuretic	7
		Antihyperlipidemic	8
		Nephroprotective	9
		Aphrodisiac	10
		Infertility Libido Cardiac diseases	11
		Intestinal disorder	12
		Antilithiatic	13
		Reno protective	14
2.	**WoodfordiaFruticosa (Dhaitiphool)**	Astringent Stimulant Anthelmintic Uterine Sedative	15

		Antibacterial	15
		Hepatoprotective	16
		Sprue, Rheumatism Dysuria Dysentery	17
		Antifertility	18
		Gastroprotective (Gastric Ulcers)	19
		Immunostimulatory	20

Sr. No	**Phytochemical**	**Reported Activity**	**Reference**
3.	**Acacia nilotica** **[Babool]**	Antioxidant Free Radical Scavenging	21
		Hypoglycaemia Bronchitis Asthma Pneumonia Meningitis	22
		Antimicrobial	23 24

		Antihypertensive Antiplasmodial Antibacterial Antifungal Antioxidant	25

3.AIM AND OBJECTIVES

Ayurvedic plants like Crataeva nurvala, Dolichos biflorus, Tribulus terrestris, Dendrophthoe falcata, Boerhaavia diffusa, Saccharum officinarum, Butea frondosa, Boerhaavia repens, Boerhaavia rependa, Homonia riparia, Centratherum anthelmintivum, Vitis venifera and Duranta repens shows very good diuretic activity. [1]

Primary health care of 88% people of world relys on traditionanl healthcare system. [26].In modern system of medicine[27] pharmacological evaluation of herbal formulation is neccessary and is a fundamental requirement of industry and other organizations dealing with ayurvedic and herbal products.[4,5]

Gokhru Kadha is an ayurvedic formulation consisting of Tribulus Terrestris and Woodfordia Fruticosa and it is manufactured by SanduPharmaceutical. It is used in several renal disorders such as Urinary calculus, urinary tract infection, oliguria, haematuria, glomerulonephritis but there is no scientific reports on the use of the selected formulation as diuretics in literature.

Hence, it is decided to conduct the systematic diuretic study on this formulation. The study will support its use as diuretics and it can be used in combination with the other allopathic drugs to decrease the dose and to have synergistic activity (If any).

3.1 OBJECTIVES OF WORK

- To review scientific literature available on the current diuretics therapies & review on pharmacology of selected formulation.
- To design, preparation and validation of metabolic cage.
- To find out diuretic effect of herbal formulation (Gokhru Kadha).
- To investigate/propose probable mechanism of action of herbal formulation.

3.2 PLAN OF WORK

1.Research study can be divided in to different phases:

Phase 1: Literature survey

Phase 2: Design and preparation of metabolic cage

Phase 3: Treatment with test drug

Phase 4: Assessment of diuretic activity of test drug with the help of various parameters.

Phase 5: Compilation of results interpretation of data

Phase 6: Paper publications

The detailed plan of work for the present investigation will be as follows

2. Pharmacological studies

2.1. Diuretic activity:

Diuretic activity will be determined by following the methods of Kau et al. (1984) [28], with minor modifications made by Benjumea et al. (2009). [29]

Two days prior to experiments, mice were acclimatized in metabolic cages with free access to food and water. The animals were fasted four hours before testing, with free access to drinking water only. All animals will be given an oral loading of normal saline (5% bow) and then following experimental design will be followed.

Acute diuretic study*:*

Freshly prepared doses of test and standard drug will be administered to test and standard animal group respectively. The control group will receive vehicle only.[30] Immediately after dosing, animals will be kept in metabolic cages for 6 hours and finally urine will be collected, measured and filtered at the end of 6 hours[30] for various biochemical estimations.

Chronic diuretic activity:

Daily doses of test and standard drugs will be given to 4-hour fasted test and standard drug animal groups respectively for 8 days. Urine volume, urinary electrolyte level will be estimated on 24 hrs. collected urine. On 8th day, additionally urinary glucose will be estimated.

Gr. No	Group (n=6)	Treatment
1	Normal control	Normal Saline (0.9% NaCl solution)
2	Treatment (Test drug)	HF (20ml/kg)
3	Treatment (Test drug)	HF (40ml/kg)
4.	Treatment (Test drug)	HF (60ml/kg)
5.	Treatment (Standard drug)	Furosemide(10mg/kg)

Table 3.1 - Experimental design.

2.3. Assessment of effect of test drug with the help of various parameters.

Following parameters will be evaluated

1. Urine volume[30,31]
2. pH [30,31]
3. Osmolality [30,31,32]
4. Electrolytes [30,31,32]
    a. Sodium,
    b. Potassium
    c. Chloride
5. Saluretic activity
6. Natriuretic activity
7. Diuretic Activity
8. Saluretic index [30,32]
    a. Sodium
    b. Potassium
    c. Chloride
9. Natriuretic index [30,32]
10. Diuretic index [30,32,33]

3.3 SCOPE OF WORK:

The polyherbal formulation (Gokhru Kadha) can be used clinically as a scientifically validated therapy for diuretic activity.

Support the use of herbal formulation in combination with other allopathic drugs to decrease dose and/or to have synergistic activity. (If any)

4.EXPERIMENTAL WORK

4.1 Experimental Animals

The Male Swiss mice were procured from Bombay Veterinary College, having body weight between 30 to 40g and used in the experiment.

Male Swiss mice were divided into group of five and six animals in each, placed in laboratory cages. The animals were acclimatized to laboratory conditions for a week before initiating the actual experimental work. They were housed under standard environmental conditions of temperature at 22 ± 1˚C under a 12-hr. light: 12 hr. dark cycle (8.00 am-20.00 pm) with free access to water and standard laboratory feed.[34]

The care and handling of mice were in accordance with the internationally access standard guidelines for use of animals.

4.2 Approval of Experimental Protocol-

The Protocol was approved by Institutional Animal Ethical Committee. **Reference No – AIKTC/SOP/IAEC/2019/03**

4.3 Metabolic Cage- Metabolic Cage was designed and fabricated at institution with the help of mechanical department.

4.4 Drugs and Chemicals-

The Gokhru Kadha, an ayurvedic formulation consisting of *Tribulus terrestris* and *woodfordia fruticosa* manufactured by Sandu Pharmaceutical Pvt. Ltd. was procured from local market.

Standard drug Furosemide (10 mg/kg) was used with 0.9% sodium chloride saline solution (vehicle) procured from local supplier.

4.5 Drug dosage administration- The Herbal formulation (HF) Sandhu's Gokhru Kadha was administered by converting the human dose to animal dose according to weight of the mice by using following formulae.

Volume of HF to be administered: Human Dose × 0.0026 → Xg/10g of mice [35]

Where X is weight of mice and 0.0026 is a conversion factor.

Standard drug furosemide (5 mg/kg) made as suspension with 0.5% w/v carboxymethyl cellulose (CMC) and administered orally as a single dose. [36]

4.6: Animal groups:

The animals will be divided in to 5 groups (6 animals per group) as follows.

Group I*:* Served as control and was administered with vehicle i.e. Saline solution.

Groups II, III and IV*:* Served as treatment group and a single dose of 20 ml/kg, 40ml/kg and 60 ml/kg of HF administered orally to group II, III and IV respectively.

Group V: Served as standard, treated with furosemide (10 mg/kg), and dissolved in 0.9% normal saline with CMC.[36]

4.7: Diuretic activity:

The Diuretic activity was determined by following the methods of Kau et al. (1984)[28] with minor modifications made by Benjumea et al. (2009) [29]

Two days prior to experiments, mice were kept in metabolic cages with free access to food and water for acclimatization. Four hours before testing, the animals were fasted, with free access only to the drinking water. All animals were given an oral loading of normal saline (5% bw) and then following experimental design was followed.

The study was conducted in acute and chronic phase.

Acute diuretic study*:*

Freshly prepared doses of test and standard drug were administered to test and standard animal group respectively. The control group will receive vehicle only [30].

Immediately after dosing, animals were kept in metabolic cages for 6 hours and finally urine was collected, measured and filtered at the end of 6 hours [30] for various biochemical estimations.

Chronic diuretic activity:

Daily doses of test and standard drugs were given to 4-hour fasted test and standard drug animal groups respectively for 8 days. Urine volume, urinary electrolyte level was estimated on 24 hrs. collected urine. On 8th day, additionally urinary glucose was estimated.

4.8 Pharmacognostic Evaluation

Pharmacognostic evaluation was performed by referring following methods [37]

Alkaloids-

The test solution (Herbal Formulation i.e. Gokhru Kadha) was added with Potassium Bismuth Iodide Solution (Dragendroff's Reagent) that gave reddish brown precipitate.

Saponins

The Froth test was used for evaluation of presence of saponins. An aqueous solution of 0.5 mg of the extract was vigorously shaken for 2 minutes in a test tube. Foam that persisted for 30 minutes and did not disappear upon warming was taken as an indication of the presence of saponin in the extract.

Flavonoids

2 mL of aqueous solution of the extract, 4 drops of 2% lead acetate solution was added. The mixture showed yellow or orange colour confirming the presence of flavonoids.

Terpenoids

One mixture of 1mL of 2,4-dinitrophenylhydrazine solutions (0.5 g) dissolved in 100 mL of 2 M HCl) was added to 2 mL aqueous solution of the extract. The formation of yellow-orange coloration indicated the presence of ketonic terpenoids.

Anthraquinones

Modified Bontrager's test: Boil the Test solution with 1 ml of Sulphuric Acid in a test tube for 5 minutes. Filter while hot. Cool the filtrate and shake with equal volume of Chloroform. Separate the lower layer of Chloroform and shake it with half of its volume of Dil.Ammonia. A rose pink to red colour is produced in the ammoniacal layer.

Tannins

Three drops of 5% ferric chloride solution was added to 1 mL of the extract solution in water. A greenish or blue coloration or precipitation was taken as an indication of the presence of tannins.

Test for glycosides (Keller–Kiliani's test)

To 0.5 g of each extract suspended in 5 mL water, 2 mL of glacial acetic acid containing one drop of ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) solution was added. This was mixed with 1 mL of concentrated H_2SO_4 and observed for a brown ring at the interface or a violet ring below the brown ring; alternatively, acetic acid was added and observed for a greenish ring above the brown ring, which gradually spread throughout this layer.

Test for Carbohydrates (Molisch Test)

In test solution, few drops of Alc. Alpha naphthol was added, then few drops of conc. Sulphuric acid through sides of test tube was added, purple to violet colour ring appeared at the junction.

4.9 Pharmacological Evaluations-

As the body excretes more urine during diuretic treatment, more electrolytes and water is lost during urination.

Hence evaluation of electrolytes present in urine is important to identify the proposed mechanism of action of the herbal formulation.

A) Evaluation of Sodium-Colorimetric method

Principle-

The present method is based on reaction of sodium with a selective chromogen (phosphonazo III) changing a colour from violet to blue in presence of the chelating agent whose absorbance varies directly as the concentration of the sodium in the test specimen.

Procedure-

The samples were analysed for sodium content using the reagent kit. The working reagent was provided readily. (Concentration of Standard-150mEql/l)

For colour development:

	Blank	**Standard**	**Test**
Colour Reagent	1000µl	1000µl	1000µl
Standard	-	10µl	-
Urine Sample	-	-	10µl

Mix & Incubate for 5 min. At RT. Measure Absorbance of sample (AT) and standard (AS) against Reagent Blank at 630 nm.

Calculation-

Sodium (mmol/l) = AT/AS × Conc. Of Standard

B) Evaluation of Potassium-Colorimetric method

Principle-

Potassium ions in a free alkaline medium react with sodium tetraphenyl boron to produce a finely dispersed turbid suspension of potassium tetraphenyl boron. The turbidity produced is proportional to the potassium concentration and react photometrically.

Procedure-

The samples were analysed for Potassium content using reagent kit. The working reagent was provided in ready to use format. (Coentration of standard-5.0 mEq/L)

For colour development:

	Blank	**Standard**	**Test**
Colour Reagent	1ml	1ml	1ml
Standard	-	20µl	-
Urine Sample	-	-	20µl

Mix & Incubate for 5 min. At RT. Measure Absorbance of sample (AT) and standard (AS) against Reagent Blank at 630 nm.

Calculation-

Potassium (mEq/l) = AT/AS × Conc. Of Standard.

C) Evaluation of Chloride-Colorimetric method

Principle-

Chloride ions react with the mercurous thiocyanate to form mercury per chlorate and thiocyanate. Thiocyanate forms a red complex with the ferric ions in presence of nitric acid.

Procedure-

The samples were analysed for Chloride content using the reagent kit. The working reagent was provided readily.

Collect 24 Hr. Urine specimen in the chloride free containers. Dilute a sample ½ in distilled water. Mix. Multiply results by 2(dilution factor) (Concentration of Standard-100mEql/l)

For colour development:

	Blank	**Standard**	**Test**
Colour Reagent	1000µl	1000µl	1000µl
Standard	-	10µl	-
Urine Sample	-	-	10µl

Mix & Incubate for 5 min. At RT. Measure Absorbance of sample (AT) and standard (AS) against Reagent Blank at 505 nm. Colour is stable for 30 min. at RT.

Calculation-

Sodium (mmol/l) = AT/AS × Conc. Of Standard × 2

D) Evaluation of Glucose-Colorimetric method

Principle-

Glucose + O_2 $\xrightarrow{\text{Glucose Oxidase}}$ Gluconic acid + H_2O_2

2 H_2O_2 + phenol $\xrightarrow{\text{Peroxidase}}$ Coloured Quinonimine Dye + 4 H_2O +amino-4 antipyrine

Procedure-

The samples were analysed for Chloride content using reagent kit. The working reagent was provided in ready to use format.

Collect 24 Hr. Urine specimen in chloride free containers. Dilute a sample ½ in distilled water. Mix. Multiply results by 2(dilution factor) (Concentration of Standard-100mEql/l)

For colour development:

	Blank	**Standard**	**Test**
Colour Reagent	1000µl	1000µl	1000µl
Standard	-	10µl	-
Urine Sample	-	-	10µl

Mix & Incubate for 5 min. At RT. Measure Absorbance of sample (AT) and standard (AS) against Reagent Blank at 505 nm. Colour is stable for 30 min.at RT.

Calculation-

Sodium (mGs/dL) = O.D. Test/O.D. STD. × Conc. Of Standard × 100

E) Evaluation of Nitrogen Urea-

Principle-

Urease hydrolyses urea to ammonia and CO_2, the ammonia formed further combines with ketoglutarate and NADH to form glutamate and NAD.The rate of oxidation of NADH to NAD is measured as a decrease in absorbance in a fixed time which is proportional to the urea concentration in the samples.

Procedure-

Mix 4 volume of Reagent 1 and 1 volume Reagent2 (i.e. 400ul Reagent 1 and 1100 ul of Reagent2). Working reagent is stable for 5 days at 20-25 ˚C and for 30 days at 28˚C.

For colour development:

	Blank	**Standard**	**Test**
Colour Reagent	1ml	1ml	1ml
Standard	-	10µl	-
Urine Sample	-	-	10µl

Mix well & read the initial absorbance A1 for the standard and Test after exactly 30 seconds. Read another absorbance A2 of the standard and test exactly 90 sec later at 340 nm. Calculate the change in absorbance ΔA for both the standard and test.

Calculation-

For Standard- $\Delta AS = A_2S + A_1S$

For Test- $\Delta AT = A_2T - A_1T$

Urea (mg/dl) = $\Delta AT / \Delta AS \times 40$

F) Evaluation of pH- pH of the urine samples was measured using pH meter

G) Evaluation of Electrolytes-

1) Osmolality [30,31,32] = [2x (urine Na) + urine K+(urinary urea nitrogen/2.8) +(urine glucose/18)]
2) Saluretic Activity[1] = Sum of Sodium and Potassium
3) Diuretic index[34] = [Urine volume of Test/Urine volume of Control]
4) Diuretic Activity[34] =[Urine volume of Test/Urine volume of Standard]
5) Saluretic index[1] , Na=[Na of test/Na of Control]; K = [K of test/K of Control]; Cl = [Cl of test/Cl of Control]
6) Natriuretic index[1] = [Natriuretic activity in test group/Natriuretic activity in control group]
7) Natriuretic Activity[1]=[Urine Sodium/Urine Potassium]

4.10 Statistical analysis-

Data are expressed as mean± S.D. (Standard Deviation.)

Statistical analysis were performed with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test[1].

5. RESULTS

5.1 Pharmcognostic Evaluation-:

Sr.No.	Parameter	Result
1.	Alkaloids	Present
2.	Flavonoids	Present
3.	Tannins	Present
4.	Steroid and Triterpenoids	Present
5.	Cardiac Glycosides	Absent
6.	Saponins	Present
7.	Carbohydrates	Present
8.	Anthraquinone Glycosides	Present

Table 5.1: Pharmacognostic Constituents

According to the obtained data; Gokhru Kadha contains the mixture of saponins, flavonoids, steroids, terpenoids etc. The salurtic activity obtained might be due to the presence of saponins that modulate renal sodium excretion. Presence of phenolic compounds, organic acids and polar compounds like flavonoids and saponins might be accountable for diuretic activity [1].

5.2 Pharmacological Evaluation:

The different parameters were analyzed for the acute and chronic study of HF in the mice, as well as the Standard drugs and control groups, are included in Tables 5.2,5.3,5.4 &5.5

Table 5.2 Effect of HF on the urine volume and urinary electrolytes and other evaluations in mice during acute study.

Groups	Dose (mg or ml/kg)	Weight (gm)	Urine output [ml/6hrs]	Na (mmol/L)	K (mEq/L)	Cl (mEq/L)	Osmolality	pH
Control	---	36.51±3.25	0.34±0.03	43.14±0.74	13.32±0.48	73.11±0.41	107.46±1.56	8.81±0.01
Std.Furosemide	10	35.17±4.02	1.42±0.16*	106.07±0.83*	43.06±1.11*	296.20±0.75*	263.55±2.27@	8.92±0.05
HF.I	20	35.55±2.23	0.55±0.04#	71.08±0.71*	21.14±0.49*	142.03±0.62*	170.43±1.54@	9.07±0.08
HF.II	40	37.68±2.65	0.97±0.07*	85.14±0.93*	33.38±0.94*	176.13±0.72*	212.92±2.10@	8.81±0.01
HF.III	60	38.13±5.59	1.31±0.19*	98.38±0.55*	39.03±0.65*	192.50±0.74*	246.50±1.24@	8.87±0.07

Values are expressed as Mean±SD, n= 6;* $P < 0.01$, # $P < 0.05$, @ $P < 0.001$ As compared to control group.

5.2.a)Urine volume and Electrolyte Excretions:

An increase in sodium level and urine volume was observed in standard and treated groups as compared to control group. K and Cl was found to be increased in standard and treated groups. The HF I produced a significant increase in urine output (# $P < 0.05$), which was much higher than that shown by the HFII , HFIII and Standard drug (* $P < 0.01$) when compared with the control group. All the groups show significant increases in electrolytes like Na, k and Cl (* $P < 0.01$) when compared with the control group.

5.2.b) Osmolality and pH:

Urine osmolality is used to measure the number of dissolved particles per unit of water in the urine. Urine osmolality is usually used to evaluate: renal function activity, polyuria and oliguria. As electrolytes excretion increased it showed highly significant increase in Osmolality (@P<0.001) in standard and Treatment groups.

Osmolality was calculated by following formulae,

Osmolality [2x(urine Na)+ urine K+(urinary urea nitrogen/2.8)+(urine glucose/18)]

The dose related response was observed and induces high Na+, K+, Cl- excretion with alkalinization of urine pH.

Table 5.3 : Effect of HF on urine volume and urinary electrolytes and other evaluations in mice during chronic study.

Groups	Dose (mg or ml/kg)	Weight (gm)	Urine output [ml/24hrs]	Na (mmol/L)	K (mEq/L)	Cl (mEq/L)	Osmolatity	pH
Control	--	38.63±3.16	0.62±0.05	76.10±1.36	25.11±1.21	156.16±0.73	185.83±1.92	8.93±0.03
Furo-semide	10	34.25±4.53	2.16±0.29*	170.03±1.42*	65.20±0.73*	400.12±0.81*	414.35±2.96@	8.81±0.06
HF.I	20	29.45±1.79	1.05±0.06*	110.08±1.43*	41.47±1.01*	160.19±0.76#	269.39±3.39@	9.14±0.03
HF.II	40	37.65±3.29	1.57±0.14*	126.10±1.12*	53.25±0.82*	173.12±0.78*	320.34±2.65@	9.42±0.08
HF.III	60	37.15±6.02	2.01±0.33*	165.17±0.93*	62.55±1.01*	220.30±0.97*	412.06±2.34@	8.07±0.05

Values are expressed as Mean±SD, n= 6;* P < 0.01 , # P < 0.05 , @ P < 0.001 As compared to control group.

5.3.a) Urine volume and Electrolyte Excretions:

An increase in sodium level and urine volume was observed in standard and treated groups as compared to control group. K and Cl was found to be increased in standard and treated groups. In standard and treated groups significant increase in urine output (* P < 0.01) was observed when compared with the control group. All the groups show significant increases in electrolytes like Na, k and Cl (* P < 0.01) when compared with the control group.

5.3.b) Osmolality and pH:

Urine osmolality is used to measure the number of dissolved particles per unit of water in the urine. Urine osmolality is usually used to evaluate: renal function activity, polyuria and oliguria. As electrolytes excretion increased it showed highly significant increase in Osmolality (@P<0.001) in standard and Treatment groups.

Osmolality was calculated by following formulae.

Osmolality [2x(urine Na)+ urine K+(urinary urea nitrogen/2.8)+(urine glucose/18)]

The dose related response was observed and induces high Na+, K+, Cl- excretion with alkalinization of urine pH.

Table 5.4 : Effect of HF on the diuretic ,saliuretic ,natriuretic index and activities evaluation in mice during Acute Study.

		Saliuretic index							
Groups	**Saliuretic Activity**	**Na**	**K**	**Cl**	**Natriuretic activity**	**Natriuretic index**	**Diuretic activity**	**Diuretic Index**	**Urine output [ml/6hrs]**
Control	56.46	1	1	1	3.27	1	--	1	0.34±0.03
Std.Furosemide	149.12	2.46	3.24	4.05	2.46	0.76	1	4.16	1.42±0.16*
HF.I	92.23	1.65	1.59	1.94	3.36	1.04	0.39	1.6	0.55±0.04#
HF.II	118.97	1.97	2.54	2.41	2.56	0.78	0.69	2.84	0.97±0.07*
HF.III	137.42	2.28	2.93	2.63	2.52	0.78	0.93	3.83	1.31±0.19*

Values are expressed as Mean±SD, n= 6;* P < 0.01 , # P < 0.05 , @ P < 0.001 As compared to control group.

5.4.a) Diuretic Activity and index with respect to urine output:

An increase in urine volume was observed in standard and treated groups as compared to control group. Hence, Diuretic Activity was found to be increased in standard and treated groups. In standard and treated groups significant increase in urine output (* P < 0.01) was observed when compared with the control group. HFII and HfIII treated groups show significant increases in Diuretic index as well as Diuretic activity which shows comparable effect as that of standard dose.

Diuretic activity was calculated by the formulae,

Diuretic activity [Urine volume of test /Urine volume of Standard]

5.4.b) Saliuretic and Natriuretic activity:

All the groups showed significant increases in electrolytes like Na, k and Cl (* P < 0.01) when compared with the control group. As saliuretic and natriuretic activity are the sum and division of some electrolytes ,electrolytes values of treated and standard groups gave increasing saliuretic activity and decreasing natriuretic activity.

Saliuretic activity was calculated by the formulae,

Saliuretic activity [Na+K]

Natriuretic activity was calculated by the formulae,

Natriuretic activity [Na/K]

5.4.c) Saliuretic and Natriuretic Index:

By comparing Treated, Furosamide and control groups with electrolytes, the saluretic and natriuretic index were calculated .The saluretic and natriuretic index of Treated groups showed significant increase as closer to the standard group.

Table 5.5 Effect of HF on the Diuretic. Saliuretic, Natriuretic index and activities evaluation in mice during Chronic Study.

		Saliuretic index							
Groups	**Saliuretic Activity**	**Na**	**K**	**Cl**	**Natriuretic activity**	**Natriuretic index**	**Diuretic Activity**	**Diuretic Index**	**Urine output [ml/24hrs]**
Control	101.21	1	1	1	3.03	1	--	1	0.62±0.05
Std.Furosemide	235.23	2.24	2.6	2.56	2.61	0.86	1	3.47	2.16±0.29*
HF.I	151.55	1.45	1.65	1	2.66	0.88	0.49	1.67	1.05±0.06*
HF.II	179.35	1.66	2.13	1.11	2.37	0.78	0.74	2.52	1.57±0.14*
HF.III	227.72	2.17	2.5	1.41	2.64	0.87	0.95	3.21	2.01±0.33*

Values are expressed as Mean±SD, n= 6;* P < 0.01 , # P < 0.05 , @ P < 0.001 As compared to control group.

5.5.a) Diuretic Activity and index with respect to urine output:

An increase in urine volume was observed in standard and treated groups as compared to control group. Hence, Diuretic Activity was found to be increased in standard and treated groups. In standard and treated groups significant increase in urine output (* P < 0.01) was observed when compared with the control group. HFII and HfIII treated groups show significant increases in Diuretic index as well as Diuretic activity which shows comparable effect as that of standard dose.

Diuretic activity was calculated by the formulae,

Diuretic activity [Urine volume of test /Urine volume of Standard]

5.5.b) Saliuretic and Natriuretic activity:

All the groups showed significant increases in electrolytes like Na, k and Cl (* P < 0.01) when compared with the control group. As saliuretic and natriuretic activity are the sum and division of some electrolytes ,electrolytes values of treated and standard groups gave increasing saliuretic activity and decreasing natriuretic activity.

Saliuretic activity was calculated by the formulae,

Saliuretic activity [Na+K]

Natriuretic activity was calculated by the formulae,

Natriuretic activity [Na/K]

5.5.c) Saliuretic and Natriuretic Index:

By comparing Treated, Furosamide and control groups with electrolytes, the saluretic and natriuretic index were calculated .The saluretic and natriuretic index of Treated groups showed significant increase as closer to the standard group.

6.DISCUSSION

Diuretics increases the rate of urine flow and sodium excretion. Also used to adjust the composition and volume of body fluids in many such clinical situations. Drug-induced diuresis is many benefits in life threatening conditions such as nephritic Syndrome, Congestive heart failure, cirrhosis, Hypertension, renal failure and pregnancy toxaemia [32].

Many diuretics have some adverse Effect on quality of life such as fatigue, Impotence, and weakness. High efficacy diuretics have the hurdles of causing increased excretion of potassium in urine.[33] Hence finding out newer drug with less adverse effect and potassium sparing activity was the need of the hour.

Gokhru Kadha is an ayurvedic herbal formulation consisting of mainly *Tribulus terrestris* and *woodfordia fruticosa* manufactured by Sandu Pharmaceutical Pvt.Ltd. It is used for several renal disorders such as Urinary tract infection(UTI), Urinary calculus, haematuria, oliguria, glomerulonephritis but the scientific reports on the use of the selected formulation as diuretics is lacking in literature.

Hence, it was decided to conduct the systematic diuretic study on this formulation. The study will support its use as diuretics and it can be used in combination with the other allopathic drugs to reduce the dose and to have synergistic activity (If any).

Gokhru kadha contains *Tribulus terrestris, woodfordia fruticosa and Acacia nilotica. Tribulus terrestris* has been extensively studied and the presence of saponins, flavonoids, alkaloids, lignan amides and cinnamic acid amides has been reported already[38.] When phytochemical evaluation of *woodfordia fruticosa* was disclosed it showed the occurence of proteins, carbohydrates and amino acids, glycosides, polyphenols, saponins, alkaloids, phytosterols, fixed oils, gums and mucilage, resins and flavonoids. [39]. Similarly, Acacia *nilotica was found to contain flavonoids, tannin, gums etc* [40]

According to the obtained data; Gokhru Kadha contains the mixture of saponins, flavonoids, steroids, terpenoids etc. Saponins presence may be responsible for saliuretic activity by regulating renal sodium excretion. Presence of phenolic compounds, organic acids and polar compounds like flavonoids and saponins might be accountable for diuretic activity [1].

The HF showed an increase in urine volume that appeared to vary with dose in acute (Table 5.2) and chronic studies (Table 5.3). The lower dose of HF did not show an appreciable effect, but, whilst the medium and high dose of the HF have produced significant effect in

acute and chronic studies. This could mostly suggest that the lower dose might represent subthreshold doses. The higher dose of HF showed comparable effect to that of standard furosemide.

The effect of the HF on water excretion was done along with urinary electrolyte excretion effect, as it showed an increased salt excretion as compared to the control group, which supports the idea that the diuretic effect of *Tribulus terrestris* was of the saluretic type in contrast to aquaretic type, which is a typical feature of the most Phyto diuretic agents [7]. The larger doses of HF did have a remarkable natriuretic effect and thus could have a favourable effect in different necessary conditions. The ratio Na^+/K^+ was calculated to get natriuretic activity. The result indicates that the HF increases sodium excretion much better than potassium, which is recognized as a very good safety profile of diuretic agents, as one of the harmful adverse effects of synthetic diuretics is hypokalaemia , with diuretics such as furosemide.

Loop diuretics like furosemide increase urinary flow rate and urinary excretion of sodium, potassium and chloride by holding back Na^+–K^+–$2Cl^-$ symporter in the thick ascending loop and by inhibiting carbonic anhydrase enzyme. The larger doses of HF used in the present study shown similar Na^+ and Cl^- excretion profile as that of standard furosemide.

Urine Osmolality is used to measure the number of dissolved particles per unit of water in the urine. As measure of urine concentration, osmolality is much accurate than the specific gravity. Urine osmolality is helpful in diagnosing urinary concentration disorders such diabetes insipidus and in assessing hydration status.Although , the assessment of any disorder involving antidiuretic hormone (ADH) will require both serum and urine osmolality to know concentrating ability of the kidney.

Urine osmolality is most probably used to evaluate- renal function, activity, oliguria and polyuria. Healthy kidneys can concentrate urine to an osmolality 4 times greater than serum. They can also dilute urine to ¼ the osmolality of serum. Patients are unable to concentrate urine having impaired renal function. As a result, urine osmolality can fall to approach that of serum, approximately 290mOsm/Kg.

A 24 hours urine osmolality must be average between 500 and 800 mOsm/Kg

The pH of urine was found to be alkaline as dose related response was obtained that also induced high Na^+, K^+, Cl^- excretion.

HF III produced alkaline urinary pH which was closer to control group and std. group as shown in Table N0-5.2, 5.3.

7. CONCLUSION

Through the outcome of this study, it is reasonable to conclude that the Gokhru Kadha possess a significant diuretic activity in mice. Thorough studies are required to determine the diuretic activity in combination with the synthetic drugs and to elucidate possible mechanism of action. Futhermore, efforts should also be taken towards identifying synergistic effect with other synthetic drugs so as to deduce the dose and adverse effects of synthetic diuretics.

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APPENDIX

ANJUMAN-I-ISLAM'S

KALSEKAR TECHNICAL CAMPUS, NEW PANVEL

Approved by : All India Council for Technical Education, Council of Architecture, Pharmacy Council of India New Delhi,
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Ref No: AIKTC/ SoP/ IAEC/ 2019/03 Date: 21st September 2019

To,
Prof. Mirza Anwar Baig
Assistant Professor,
School of Pharmacy, AIKTC,
New Panvel-410206.

This is certify that the project title "**Evaluation of Herbal Formulation for Diuretic Activity in Experimental Animals.**" (Proposal No:AIKTC/SoP/IAEC/2019/03) has been approved by the IAEC, School of Pharmacy, Anjuman-I-Islam's Kalsekar Technical Campus, Panvel-410206. The said proposal is approved with following terms and conditions:

1. **Name of experimental model (Species):** Swiss mice
2. **No of animal for the study:** 30
3. **Duration of the Project:**
   a. **Number of months** : 6 month
   b. **Date of initiation (proposed)** : 1st December 2019
   c. **Date of completion** : 1st June 2020

(Chairperson, IAEC)
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I/c Dean, SoP
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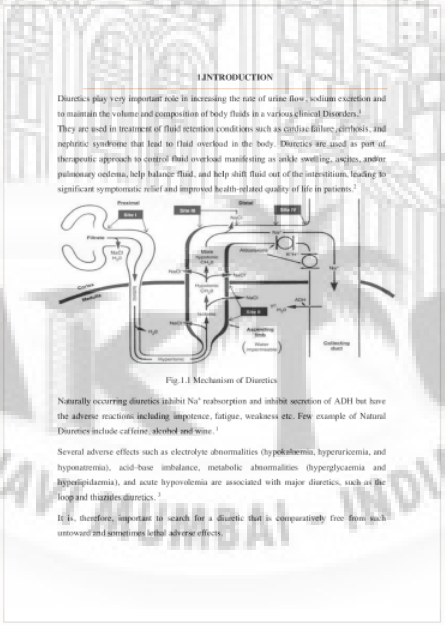

1.INTRODUCTION

Diuretics play very important role in increasing the rate of urine flow , sodium excretion and to maintain the volume and composition of body fluids in a various clinical Disorders.[1]
They are used in treatment of fluid retention conditions such as cardiac failure, cirrhosis , and nephritic syndrome that lead to fluid overload in the body. Diuretics are used as part of therapeutic approach to control fluid overload manifesting as ankle swelling, ascites, and/or pulmonary oedema, help balance fluid, and help shift fluid out of the interstitium, leading to significant symptomatic relief and improved health-related quality of life in patients.[2]

Fig.1.1 Mechanism of Diuretics

Naturally occurring diuretics inhibit Na^+ reabsorption and inhibit secretion of ADH but have the adverse reactions including impotence, fatigue, weakness etc. Few example of Natural Diuretics include caffeine, alcohol and wine.[3]

Several adverse effects such as electrolyte abnormalities (hypokalaemia, hyperuricemia, and hyponatremia), acid–base imbalance, metabolic abnormalities (hyperglycaemia and hyperlipidaemia), and acute hypovolemia are associated with major diuretics, such as the loop and thiazides diuretics. [3]

It is, therefore, important to search for a diuretic that is comparatively free from such untoward and sometimes lethal adverse effects.

Evaluation of Herbal Formulation for Diuretic Activity in Experimental Animals.

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