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PHYTOCHEMICAL STUDIES AND ANTIUROLITHIATIC ACTIVITY OF *VITEX NEGUNDO* LINN ROOT EXTRACTS

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ABSTRACT

Background: Vitex negundo has many uses in Ayurveda, Homeopathy, and Allopathy to treat several diseases like venereal diseases, Urinary problems, cough and fever, asthmatic pain, female reproductive problems. **Purpose:** This study aimed to perform the comparative phytochemical study, chromatographic profiling, and Antiurolithiatic activity of Vitex negundo linn root extracts. **Methods:** Using maceration extraction, Successive reflux condensation extraction processes were used to prepare petroleum ether, methanol, aqueous, and alcoholic extracts. Preliminary phytochemical study were carried out in plant parts extract. We performed chromatographic profiling of Vitex negundo by using GC-MS. About 18 phytochemicals in Vitex negundo plant root were identified and quantified by using GC-MS. Antiurolithiatic activity was performed to study urolithiasis inhibition potency of root extracts of the selected plant. **Results:** The percentage yield of petroleum ether, methanolic and aqueous extract of Vitex negundo Linn was found to be 1.42 % w/w,0.85%w/w and 1.14% w/w respectively. The Rf value of 0.76, 0.69,0.95,0.61,0.69,0.87 and 0.95 may be due to the presence of flavonoids, alkaloids, tannin, Glycosides The urine samples of normal and treated animals were collected on 14th and 28th day and a comparative analysis has revealed that there was significant increase in the volume & pH of urine in the animals treated with methanolic extract. This study proves that there was significant decrease in calcium oxalate crystals by methanolic root extracts against ethylene glycol induce urolithiasis model in swiss albino rats. It is interesting to note from the GC MS results that the presence of biomolecules such as Dibutyl malate, Heptaethylene glycol monododecyl ether, Suc-L-Phe-OH4-Nitrophenyl, Ajmalicine, alpha-Tocotrienol, Tyroscherin, Caryoptin, Bruceantin, Unii-0E0K1H745W, 4-O-MePdd, Bevirimat, Beta-Carotene, Phytoene, Allochenodeoxycholic acid, Calamin, 3 (Benzylnonanoylaminomethyl) androsterone, Methyl betulinate,Stearoyldelicone etc. correspond well with the reported medicinal roles of Vitex negundo Linn. **Conclusion:** As per present study, The selected plant part of Vitex negundo Linn could be helpful for generating formulations for kidney stone reduction.

KEYWORDS: Urolithiasis, Vitex, root, phytochemical, Medicinal plants.

INTRODUCTION

India is endowed with a rich wealth of medicinal plants which have been a valuable source of natural products for maintaining human health. A large number of these medicinal plants are used in various formulations for the treatment of several disease caused by microbes. Medicinal plants constitute an effective source of traditional and modern medicines. Ayurveda a system of Indian traditional form of alternative medicines.Natural sources of medicinal plants are often unable to meet demand for popular herbal products. In this paper we report phytochemical and pharmacological investigation of antiurolithiatic potency of a plant known as Nirgundi (Vitex negundo L.; Verbenaceae) The present study deals with the Comparitive phytochemical and pharmacological studies of medicinal plant Vitex negundo. L. The major objectives of this study are, the study of phytoconstitutes present in various extract of plant Herbal medicine has become an important part of our health care systems. There is a great demand for herbal medicine in developed as well as developing countries like India, because of their wide range of biological activities, higher safety of margin than the synthetic drugs and lesser costs. Phytochemistry describes the large number of secondary metabolic compound found in plants. Many of these are known to provide protection against diseases. They also exhibit a number of protective functions to plants and animals. Medicinal plants rich in phytochemicals have been used for centuries in the treatment and prevention of diseases. Traditional systems of medicinal, different parts of plant is used in the treatment of various healthailiments.[1]

MATERIALS AND METHODS

Collection of Samples

Plant was collected from P.R.C.O.P campus, Loni B.K and authenticated at the dept of Botany and research center PVP college, Loni.

Preparation of Extracts

The plant material (100 g) was extracted with 350ml of each solvent using a reflux condenser apparatus. By using successive method then after three extracts were found (petroleum ether, methanol & aqueous) these extracts were concentrated in a rotary evaporator at reduced pressure.[2]

Fig No. 01: Extraction process of *Vitex negundo* Linn root.

Phytochemical analysis

Preliminary phytochemical study

The extract was screened for various constituents (alkaloids, saponins, tannins, steroids, flavonoids, glycosides) using standard protocol.

Quantitative Estimation

Quantitative Evaluation were estimated by using following methods.

Estimation of total phenol content

Folin-Ciocalteu assay method was used for the determination of the total phenol content. The reaction mixture consists of 1 ml of extract and 9 ml of distilled water was taken in a volumetric flask (25 ml). One millilitre of Folin-Ciocalteu phenol reagent was treated to the mixture and shaken well. After 5 minutes, 10 ml of 7 % Sodium carbonate (Na2CO3) solution was treated to the mixture. The volume was made up to 25 ml. A set of standard solutions of gallic acid (20, 40, 40, 60, 80 and 100 µg/ml) were prepared in the same manner as described earlier. Incubated for 90 min at room temperature and the absorbance for test and standard solutions were determined against the reagent blank at 550 nm with an Ultraviolet (UV) /Visible spectrophotometer. Total phenol content was expressed as mg of GAE/g of extract.

Estimation of total flavonoid content

Total flavonoid content was measured by the aluminium chloride colorimetric assay. The reaction mixture consists of 1 ml of extract and 4 ml of distilled water was taken in a 10 ml volumetric flask. To the flask, 0.30 ml of 5 % sodium nitrite was treated and after 5 minutes, 0.3 ml of 10 % aluminium chloride was mixed. After 5 minutes, 2 ml of 1M Sodium hydroxide was treated and diluted to 10 ml with distilled water. A set of reference standard solutions of quercetin (20, 40, 60, 80 and 100 µg/ml) were prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 510 nm with an UV/Visible spectrophotometer. The total flavonoid content was expressed as mg of QE/g of extract.

Estimation of tannin content

The tannins were determined by Folin - Ciocalteu method. About 0.1 ml of the sample extract was added to a volumetric flask (10 ml) containing 7.5 ml of distilled water and 0.5 ml of Folin Phenol reagent, 1 ml of 35 % Na2CO3 solution and dilute to 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 min. A set of reference standard solutions of gallic acid (20, 40, 60, 80 and 100 µg/ml) were prepared in the same manner as described earlier. Absorbance for test and standard solutions were measured against the blank at 725 nm with an UV/Visible spectrophotometer. The tannin content was expressed in terms of mg of GAE /g of extract.

Determination of Alkaloid content

The plant extract (1mg) was dissolved in dimethyl sulphoxide (DMSO), added 1ml of 2 N HCl and filtered.This solution was transferred to a separating funnel, 5 ml of bromocresol green solution and 5 ml of phosphate buffer were added. The mixture was shaken with 1, 2, 3, and 4 ml chloroform by vigorous shaking and collected in a 10-ml volumetric flask and diluted to the volume with chloroform. A set of reference standard solutions of atropine (20, 40, 60, 80 and 100 µg/ml) were prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 470 nm with an UV/Visible spectrophotometer. The total

alkaloid content was expressed as mg of AE/g of extract spectrophotometer. The total alkaloid content was expressed as mg of AE/g of extract.[4]

Fig No. 02: Dilutions for quantitative estimation of *Vitex negundo* Linn extracts.

Thin Layer Chromatography

Each concentrated extract was spotted on a normal phase plate previously activated at 110 ^{0}C for 2hrs, using a capillary tube. The plate was developed using mobile phase for petroleum ether, methanolic and aqueous extracts The Retardation factor (Rf) was determined using this formula:

$$Rf = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent}}$$

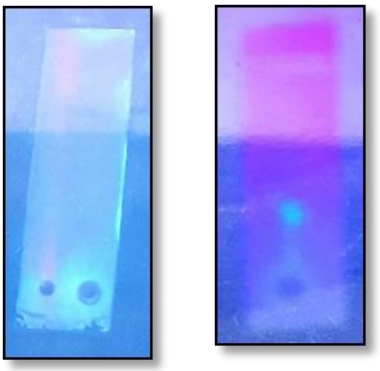

Fig No. 03: TLC study of *Vitex negundo* Linn extracts.

Column chromatography

Column was packed with slurry of silica gel (mesh size, 60-120) with chloroform. Then dried Methanol extract (1 gm) of vitex negundo linn was first dissolved in Methanol and carefully applied by pipette at the top of prepared column. Immediately after application of sample, a gradient of Chloroform and Methanol (mobile phase) was used as eluant to collect fractions of Methanol extract of vitex negundo linn. The column was run with a gradient of Chloroform : Methanol : ethyl acetate (7 : 3: 6) followed by fractionation with different organic solvents such as chloroform, methanol & ethyl acetate to separate different components from methanolic extract following the technique of liquid liquid separation. As polar compounds would come in polar solvents, those fractions which were found to be rich with phtytoconstituents with were analyzed by phytochemical study and spectral analysis these fractions were subjected to GCMS Profiling for active constituents.[5]

GC–MS chromatographic profiling of Vitex negundo

The detailed peak identification is shown in Fig. 09, and retention time, compound name, molecular formula, and peak % are given in Table No. 06.[6]

Pharmacological study

Animals

Wistar rats (150-200 g) of either sex were used. Animals were housed ingroups of six under standard laboratory conditions of temperature (25± 20 C) and 12/12 h light/dark cycle. They were provided with standard pellets and tap water ad libitum. The study protocol was approved by the Institutional Animal Ethical Committee (IAEC) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

Evaluation of antiurolithiatic activity of *Vitex negundo* Linn root extracts on ethylene glycol (0.75% v/v) induced albino rats

Ethylene glycol (0.75% v/v) induced urolithiasis model was used to study the antiurolithiatic activity in male Wistar albino rats. The animals were divided into six groups containing six each. The group I served as control and fed with normal rat food and water ad libitum. Group II to VI received ethylene glycol (0.75% v/v) orally in drinking water from day 1 to day 28 for the induction of renal calculi (day 1 to day 14-induction period). Group II served as disease induced group. Group III received reference drug Cystone (750 mg/ kg b.w) from 14th day to 28th day of calculi induction. Group IV received Petroleum ether 200 mg/kg b.w. from 14th day to 28th dayof calculi induction, group V received Methanolic extract 200 mg/kg b.w from 14th day to 28th day of calculi induction. Group VI received Aqueous extract 200 mg/kg b.w from 14th day to 28th day of calculi induction. Urine analysis was carried out at the end of the study. Urine samples were collected from all the animals on 14th and 28th day.

Physical changes in experimental animals like weight of the animals and changes in urine volume were also monitored from day 1 to day 28. Urine volume was measured on day 14 and day 28.

Statistical analysis

The biochemical results were expressed as mean ± SEM. Statistical significance of the observations from urine analysis was calculated using one way analysis of variance test (ANOVA), followed by Dunnett's t-test, value less than $p < 0.05$ were considered as statistically significant.[7,8]

RESULTS

Extractive value of Vitex negundo Linn root

Table No. 1: Extractive values Results of extractive values of different solvents.

Successive Extraction		Wt. of sample (g)	Wt. of extract (g)	Extract%
	Petroleum ether	10-12	5	1.42
	methanol		3	0.85
	Aqueous		4	1.14

Physicochemical parameters

The comparative analysis results of physicochemical parameters for Vitex negundo root are tabulated in **Table 2**.

Table No. 2: Physicochemical analysis.

Parameters	Observations
Total ash (% w/w)	12.5%
Acid insoluble ash (% w/w)	5%
Water soluble ash (% w/w)	5%
Water soluble extractive (% w/w)	16%w/w
Alcohol soluble extractive (% w/w)	10.56 %w/w
Loss on drying at 105 ◦C (% w/w)	12%

Preliminary phytochemicals screening

Table 3: The phytochemical analysis results of petroleum ether, methanol & aqueous extracts of roots of *Vitex negundo* Linn.

Phytocostituents	Petroleumether	Methanol	Aqueous extract
Favonoids	_	+	+
Saponoins	_	_	+
Alkaloids	_	+	+
Glycosides	+	+	_
Tannin	_	+	+
Phenols	_	_	_
Carbohydrates	+	_	_
Steroids	_	+	_

Quantitative estimation

Table 4: Total phenol, alkaloid, flavonoid and tannin contents in *Vitex negundo* Linn root extracts.

Phytochemical Content	Aqueous	Alcoholic
Total Phenol Content (mg of GAE/g of extract)	15.92	76.44
Total alkaloid Content (mg of GAE/g of extract)	26.13	192.04
Total flavonoid Content (mg of GAE/g of extract)	170	300.55
Total tannin Content (mg of GAE/g of extract)	143.18	61.36

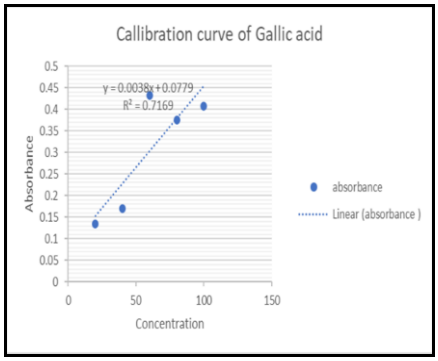

Fig No.05: Calibration curve of gallic acid.

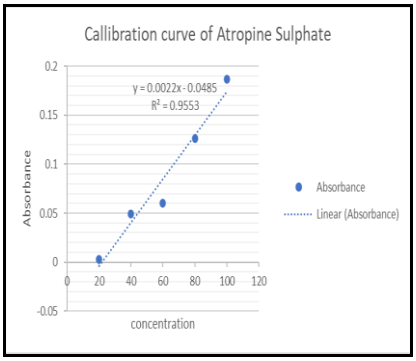

Fig No. 06 callibration curve of Atropine sulphate.

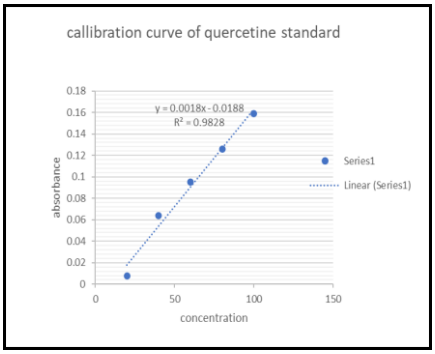

Fig No.07: Calibration curve of quercetin standard.

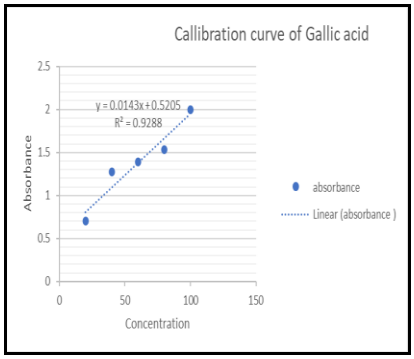

Fig No.08 calibration curve of Gallic acid.

All the calibration graphs showed that strong positive linear correlation (r) which is close to +1. These graphs indicate that as the value of concentration increases, values for absorbance also increase. Both extracts were made known less content of phenol both extracts are having presence of alkaloids, flavonoids & tannins.

Thin layer chromatographical study

Table No. 5: Rf values of phytochemicals of *Vitex negundo* Linn extracts.

Sr. No	Phytochemicals	Mobile Phase	Rf Value		
			Petroleum ether	Methanol	Aqueous extract
1	Alkaloids	Toluene: Ethyl Acetate: Diethylamine (70:20:10)	0.23	0.95	o.61
2	Glycosides	Methanol:distilled Water: Chloroform (35:10:65)	0.95	0.84	0.69
3	Flavonoids	Toluene: Ethyl Acetate (9:1)	0.95	0.76	0.69
4	Tannins	Toluene: Ethyl Acetate: Acetone (3:2:1)	0.23	0.69	0.87
5	Steroids	Toluene: Ethyl Acetate (9:1)	0.23	0.38	0.95
6	Saponin	Toluene: Ethyl Acetate (9:1)	0.32	0.38	0.95

The GC MS results of methanolic fraction of *Vitex negundo* Linn

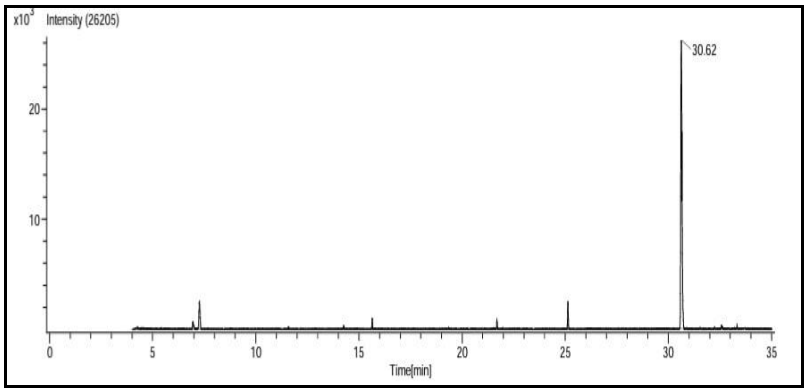

Fig No 9.1: The GC MS results of sample A from methanolic fraction.

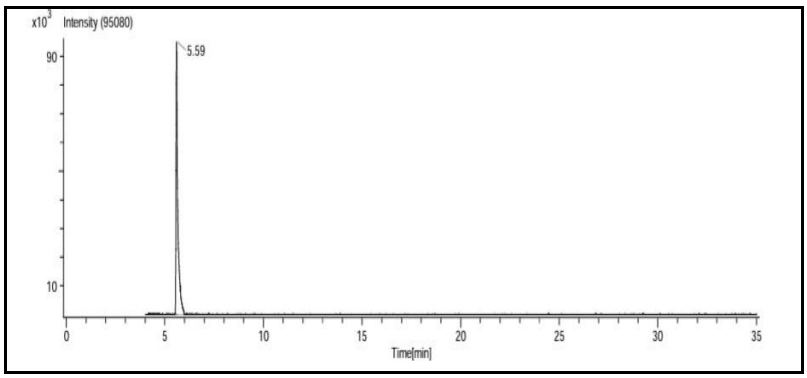

Fig No 9.2: The GC MS results of sample B from methanolic fraction.

Fig No 09: GC-MS chromatogram of, methanolic fraction of *Vitex negundo* Linn root.

Table No.06: The GC MS results of samples collected from methanolic fraction of *Vitex negundo* Linn root.

Sr. No.	RT	Compound	Mol. Formula	Percent Peak Value	Medicinal Properties
1.	5.59	Dibutyl malate	C12H22O5	43.9	Anti-inflammatory, Anti-Oxidant, and Anti-Fungal properties, and it is also known to have anti-cancer activity.
2.	5.59	Heptaethylene glycol monododecyl ether	C26H54O8	43.9	It can reduce the surface tension of aqueous solutions, increase the solubility of hydrophobic molecules, anti-inflammatory effect
3.	7.27	Suc-L-Phe-OH4-Nitrophenyl	C19H18N2O7	7.27	Promote weight loss, reduce chronic pain and protect against depression.
4.	7.27	Ajmalicine	C21H24N2O3	7.27	Antihypertensive, Sedative, Anxiolytic, Antiproliferative, and apoptotic activities. It has also been found to modulate gene expression, and to have Antioxidant, Anti-Inflammatory, and Anticonvulsant activities.
5.	7.27	alpha-Tocotrienol	C29H44O2	7.27	Neuroprotective agent, a ferroptosis inhibitor It is a vitamin E.
6.	9.65	Tyroscherin	C21H35NO2		Antineoplastic antibiotic
7.	9.65	Caryoptin	C26H36O9		Anthelmitic, hallucinogenic agent
8.	9.65	Bruceantin	C28H36O11		Antineoplastic antibiotic, antiamoebic and Antimalarial Activity.
9.	9.65	Unii-0E0K1H745W	C28H41NO2		Antineoplastic activity
10.	21.69	4-O-MePdd	C41H66O8		
11.	21.69	Bevirimat	C36H56O6	1.07	Protease inhibitor,
12.	21.69	Beta-Carotene	C40H56	1.07	Reduce inflammation, improve immune function, and enhance skin health.
13.	21.69	Phytoene	C40H64	1.07	Antioxidant, anti-inflammatory, and anti-cancer
14.	25.13	Allochenodeoxycholic acid	C24H40O4	3.96	Regulate lipid metabolism, and improve insulin sensitivity, anti-tumor effects
15.	25.13	Calamin	C27H36O10	3.96	Promote the healing of damaged skin.
16.	25.13	3-(Benzylnonanoylaminomethyl)androsterone (Compound)	C36H55NO3	3.96	
17	30.62	Methyl betulinate	C31H50O3	86.3	It induces melanogenesis
18	30.62	Stearoyldelicone	C33H54O3	86.3	Antimicrobial

Pharmacological evaluation of *Vitex negundo* Linn root extracts

Table No 07: Effect of vitex negundo linn extract on urine volume, and pH in the rats, Body weight of animal

Group	Change In Body Weight (Gm)	Volume of urine (ml)	pH
Control	96.6 ±5.69	2.08±0.15	7.1 ± 0.16
Induced (Ethylene glycol 0.75%v/v)	92±4.02	1.16±0.14	6.5 ± 0.24
Standard (Cystone 750mg/kg)	94.36±2.79	2.6±0.14	7.5 ± 0.08
Pet.ether plant extract (200mg/kg)	92.48±4.8	1.14±0.18	6.8 ± 0.05
methanolic plant extract (200mg/kg)	95.02±6.21	2.1±0.13	7.15 ± 0.16
Aqeoues plant extract (200mg/kg)	94.06±4.98	1.18±0.08	7.0 ± 0.08

Each value represents the mean ± S.E.M. of six rats, *P < 0.05, Compared to normal saline-treated group, One-way analysis of variance (ANOVA) followed by “Dunnett’s test.”

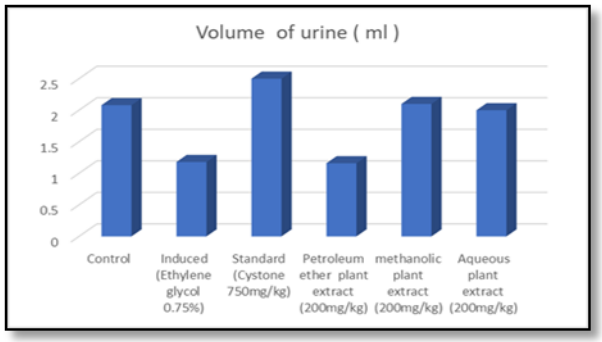

Fig No. 10 Volume of urine in ml.

Effect of Vitex negundo linn extract on urine parameters in control and experimental animals (All values represent mean ± S.E.M, N = 6, *P < 0.05, compared to control Group I, N = 6, **P < 0.05, compared to ethylene glycol Group II, One-way analysis of variance (ANOVA) followed by "Dunnett's test.")

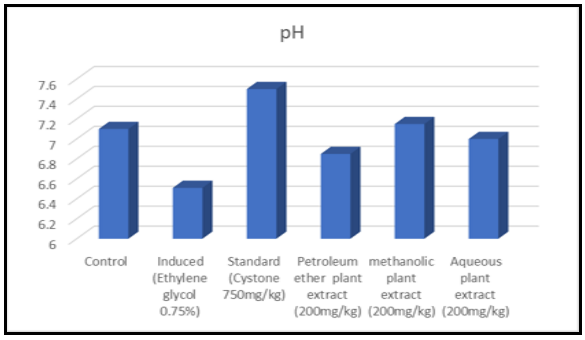

Fig No. 11 pH of urine.

Effect of Vitex negundo linn extract on urine parameters in control and experimental animals (All values represent mean ± S.E.M, N = 6, *P < 0.05, compared to control Group I, N = 6, **P < 0.05, compared to ethylene glycol Group II, One-way analysis of variance (ANOVA) followed by "Dunnett's test.")

Microscopical analysis of animal urine

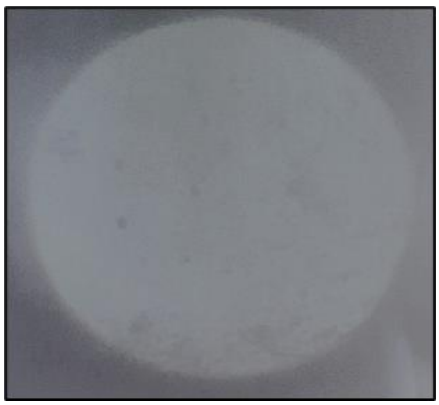

Fig No 12.1:Normal group.

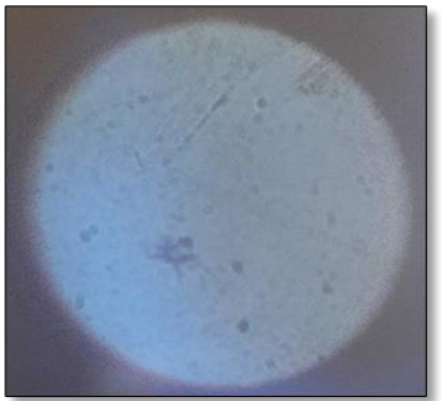

Fig No 12.2 Control group.

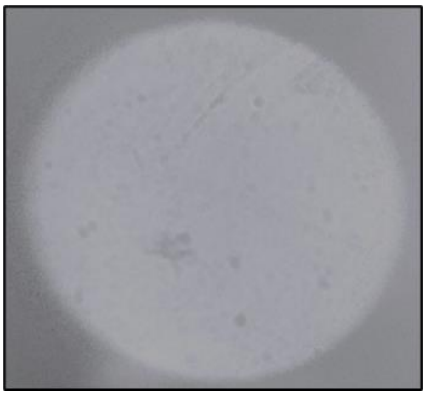

Fig No 12.3: Standard group.

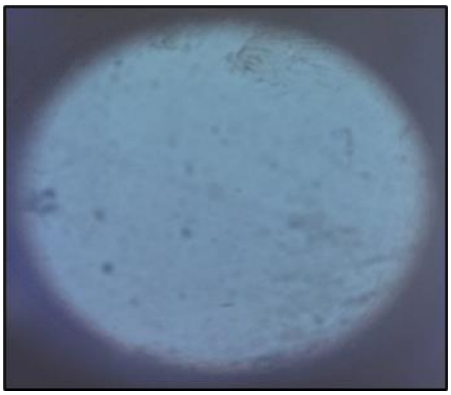

Fig No 12.4: Sample 1 treated group.

Fig No 12.5: Sample 2 treated group.

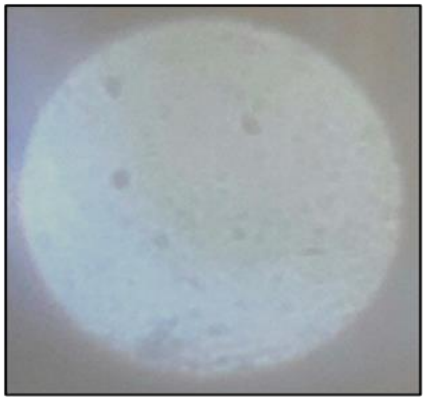

Fig No 12.6: Sample 3 treated group.

DISCUSSION

Formation of kidney stones is a complex process and involves a series of biological events that are most likely triggered by genetic susceptibility together with dietary factors and lifestyle changes. Several in vivo animal models have been developed to investigate the mechanisms involved in the formation of urinary stones. However, rat model has been widely used for the study of CaOx deposition in the kidneys, a process that mimics the etiology of kidney stone formation in humans. Consequently kidney stones formation was induced in wistar albino rats by ethylene glycol in drinking water model in the present study. The percentage yield of petroleum ether, methanolic and aqueous extract of Vitex negundo Linn was found to be 1.42 % w/w,0.85%w/w and 1.14% w/w respectively. The petroleum ether extract was dark brown in colour with a thick viscous consistency. The aqueous extract also has a thick viscous consistency and brown in colour. Preliminary Phytochemical screening of extracts revealed the The Rf value of 0.76, 0.69,0.95,0.61,0.69,0.87 and 0.95 may be due to the presence of flavonoids, alkaloids, tannin, Glycosides When viewed under UV at 366m and visible light after development in the mobile phases The Rf value of 0.9, 0.23, 0.32 and 0.38 may be due to the absence of flavonoid, alkaloid, tannin, steroids, saponin The extract also showedThe Rf may be due to the presence of different active principle might be responsible for the therapeutic activity.

The urine microscopy revealed that concentration of various ions was altered drastically in urine after the treatment with the isolated compounds. The urine sample of normal group of rats showed absence of microcrystals; the urine sample of disease induced rats showed the presence of microcrystals whereas the urine sample of treated rats showed the presence of small fragments. This creates a favorable environment for the nucleation and crystal formation in animals whereas a significant decrease in the level of microcrystals observed in the urine sample of the animals treated with the methanolic extract has resulted in the reduction of crystal formation. The urine samples of normal and treated animals were collected on 14th and 28th day and a comparative analysis was carried out for weight of the animals, volume of urine, pH of urine which were monitored. This analysis has revealed that there was significant increase in the volume & pH of urine in the animals treated with methanolic extract. The comparative analysis of the weight of animals has revealed that there was an increase in the weight of treated animals It is interesting to note from the GC MS results that the presence of biomolecules such as Dibutyl malate, Heptaethylene glycol monododecyl ether, Suc-L-Phe-OH4-Nitrophenyl, Ajmalicine, alpha-Tocotrienol, Tyroscherin, Caryoptin, Bruceantin, Unii-0E0K1H745W, 4-O-MePdd, Bevirimat, Beta-Carotene, Phytoene, Allochenodeoxycholic acid, Calamin, 3 (Benzylnonanoylaminomethyl) androsterone, Methyl betulinate,Stearoyldelicone etc. correspond well with the reported medicinal roles of Vitex negundo Linn.

Some of the compounds are present in large quantities such as Dibutyl malate (43.9), Ajmalicine (7.27), alpha-Tocotrienol (7.27), Caryoptin, Bruceantin, Bevirimat (1.07), Beta-Carotene, Allochenodeoxycholic acid (3.96), Calamin (3.96), 3-(Benzylnonanoylaminomethyl) androsterone(3.96), Methyl betulinate (86.3), Stearoyldelicone (86.3) which indicate important biological roles. The biological roles of some compounds present in small quantities such as Bruceantin, Bevirimat, Beta-Carotene, Phytoene they have important medicinal roles

CONCLUSION

The results of the present investigation on the evaluation of antiurolithiatic activity of Vitex negundo root extracts in rat models of lithiasis have led to the following conclusions. In these studies methanolic extract showed significant reduction in calcium oxalate stone in urine & an increase in the urinary volume and a restoration of normal urine volume. Methanolic extract is more potent than petroleum ether, aqueous extract in the urolithiasis rat models. Flavonoids, phenols, triterpenoids, terpenoids, steroids, carotenoids and saponins present in the extract may be responsible for antiurolithiatic activity of the extracts

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