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ANTIHYPERTENSIVE AND RENOPROTECTIVE EFFECT OF DIFFERENT FRACTIONS OF WHOLE PLANT *LIPPIA NODIFLORA* LINN. ON UNINEPHROCTIMIZED DOCA-SALT HYPERTENSIVE RATS.

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ABSTRACT

Lippia nodiflora Linn a member of Verbenaceae family, is used in traditional and Unani system of medicine because of its antispasmodic, hair afflictions, anti-inflammatory, analgesic and antipyretic, antibacterial, anti *Helicobacter pylori* activity, hypotensive activity, antinociceptive and antifungal. The purpose of the study is to check the efficacy of different fractions of whole plant *Lippia nodiflora* on uninephrectomized DOCA-salt hypertensive rats. Dried and powdered whole plant was extracted with chloroform, ethyl acetate, methanol and water and administered in doses of 500 mg/Kg p.o. Preliminary phytochemical analysis of plant reveals presence of flavonoids, alkaloids, triterpenes, glycosides and sterols. Data was analysed by one way analysis of variance (ANOVA) followed by Tukey's test. Among all these extracts, methanolic extract was found to be effective as it reduced the systolic blood pressure significantly. We have also checked the creatinine level as a renal marker; histopathology of kidney also confirmed the biochemical findings. This study supports the antihypertensive role and renoprotective effect of methanolic fractions.

KEYWORDS: ***Lippia nodiflora*, Uninephrectomized, DOCA-salt, antihypertensive**

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INTRODUCTION

It has been long recognized that hypertension is an important risk factor for cardiovascular diseases and mortality[1]. Traditionally high burden of hypertension and its adverse consequences has been mistakenly thought to be affliction of only economically developed countries [2]. However the studies over the past two decades reported that majority of people in many economically developing countries have blood pressure above the level considered optimal with high prevalence of hypertension present [3, 4, and 5]. Recent publication from Global Burden of Diseases (GBD) study has placed a renewed focus on heavy toll high blood pressure is having in all regions of the world [6, 7]. Deaths due to hypertension arise from cerebrovascular and cardiovascular complications such as stroke, end-stage renal disease, congestive heart failure, myocardial infarction, and cardiac arrest. Conventional antihypertensive are usually associated with many side effects. About 75 to 80% of the world population use herbal medicines, mainly in developing countries, for primary health care because of their better acceptability with human body and lesser side effects. In the last three decades, a lot of concerted efforts have been channelled into researching the local plants with hypotensive and antihypertensive therapeutic values. The antihypertensive effects of some of these medicinal plants have been validated and others disproved. However, ayurvedic knowledge needs to be coupled with modern medicine and more scientific research needs to be done to verify the effectiveness, and elucidate the safety profile of such herbal remedies for their antihypertensive potential[8].*Lippia nodiflora* Linn. (Verbenaceae) is commonly creeping perennial herb with small flowers known as frog fruit. It is known as *Jalpipli* in Sanskrit. It is found throughout warmer parts of India, Srilanka, Baluchistan, and Africa. It is common in wet places, along irrigation channels, canal edges and river banks. It is a creeping, prostate, much branched perennial herb with branches spreading profusely and rooting at the nodes [9]. Akhtar in 1993 reported hypotensive potential of this plant on Pentothal sodium anesthetisized rats. *Lippia nodiflora* contains flavonoids, sugars, sterol, an essential oil, resin, non glucosidal bitter substance, tannin, large amount of potassium nitrate and other constituents. Previous phytochemical analysis of this plant reveals the presence of isolated compound such as lippiflorin A and B, Nodiflorin A and B, alkaloids, essential oils, resins, stigmasterol,β-sitosterol, sulphates of neptin, jeceosidin, hispidulin 6-hydroxy luteolin a new Triterpenoids Lippiacin and a new steroid 4',5'-dimethoxybenzoloxystigmasterol has been isolated from the aerial part of the plant [10,11]. Several workers have reported many pharmacological properties including antispasmodic, hair afflictions, anti-inflammatory, analgesic and antipyretic, antibacterial, anti *Helicobacter pylori* activity, hypotensive activity, antinociceptive and antifungal. The aerial parts are used as anodyne, antibacterial, diuretic, parasiticide, refrigerant, febrifuge and cooling. According to traditional uses and Unani system of medicine the plant is acrid, hot and dry; diuretic, maturant, useful in fevers and cold, astringent to bowels, stomachic, used in lack of bowel movements, pain in knee joints and in lithiasis. The objective of present study is to fractionate the different constituents present in plant in different solvent extract and find out antihypertensive efficacy of these extracts[12,13,14,15] .

MATERIALS AND METHODS

(I)Plant material

Whole plant, *Lippia nodiflora* was collected from Botanical Garden at Shri Bapalal Vaidya Botanical Research Centre of Biosciences, Department of Biosciences, Veer Narmad South Gujarat University, Surat, Gujarat, India. The plant was identified by Dr. M. N. Reddy, Dept of Biosciences, VNSGU,Surat. The voucher specimen was deposited in herbarium of the Biosciences Department of VNSGU, Surat.

(II) Preparation of extract

The whole plant was washed under running tap water followed by distilled water and dried at 40^0C in the oven for 3 days. The dried plant was then pulverized into a fine powder that

passed through a 30-mesh sieve and stored for the future use. The ground plant material was subsequently extracted with different solvent using Soxhlet apparatus. The resulting crude extracts were filtered by passing through a Whatman No. 3 filter paper followed by concentrating in vacuum at 40^0C using a rotary evaporator and freeze drying. The freeze dried samples were suspended in 0.2% agar solution and mixed thoroughly for giving the extract orally to animals[16].

(III) Reagents and chemicals

DOCA (Deoxycarticosteroneacetate) was obtained from Sigma Aldrich, USA. Methanol, chloroform, ethyl acetate, toluene and hexane were of analytical grade and purchased from Merck. The TLC silica plates pre-coated with silica gel 60 F254 were purchased from Merck.

(IV) Animals

Albino Wister rats (150-200 gm) of either sex were obtained from the animal house, Department of Pharmacology, Maliba Pharmacy College, Tarsadi, Bardoli, Surat, Gujarat, India. Animals were housed (3 rats/cage) in polypropylene cages lined with husk, renewed every 24hr under a 14:10 hr of light/dark regime and had free access to tap water and food. The rats were fed on a standard pellet diet. All the protocols were approved by the Institutional Animal Ethical Committee (IAEC) (Protocol No. MPC/04/2012) and conducted according to the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiment on Animals) Rats of either sex were divided in to eight groups of 6 animals (n=6) each. Rats were allowed to acclimatize to the experimental room conditions for a period of six days.

(V) Surgical technique for unilateral nephroctomy

Left kidney was removed from all the rats by anaesthetizing with Ketamine (70 mg/kg i.p) and diazepam (5 mg/kg s.c). The kidney was visualized by a left lateral abdominal incision, and the left renal artery and ureter were ligated by silk thread, followed by the removal of left kidney. The muscle and skin layer (incision site) were sutured. After uninephrectomy, rats were allowed water *ad libitum*. Whole procedure was carried out in highly aseptic conditions [17].

(VI) Induction of Hypertension

After one week of recovery period all uninephrectomized animals were given 1% NaCl in the drinking water with weekly twice subcutaneous injection of DOCA-Salt (25 mg/kg body weight in olive oil) for four consecutive weeks (DOCA-salt hypertensive rat). The rats were then; randomly divided into different groups each comprising of six animals [18].

(VII) Experimental design

The animals were divided in to eight groups of six animals each and treated as shown in Table: 1

Table 1
Group distribution of animals

N0	Name of group	Treated with	Dose	Route
I	Control group	Vehicle	-	-
II	Sham operated group	Vehicle		
III	Disease control group	DOCA-salt + NaCl	25mg/kg body wt+1%	
IV	Positive control group	DOCA salt + NaCl+ standard anti-hypertensive drug-ramipril	25mg/kg body wt +1%+1mg/kg of body wt [16]	Oral
V	T1	Methanolic extract	500mg/kg of body wt	Oral
VI	T2	Chloroform extract	500mg/kg of body wt	Oral
VII	T3	Ethyl acetate extract	500mg/ kg of body wt	Oral
VIII	T4	Water extract	500mg/kg of body wt	Oral

T1=treated with methanolic extract, T2=treated with chloroform extract, T3=treated with ethyl acetate, T4=treated with water extract.

Hypertension was developed within one month after the treatment of DOCA-salt. Rats were treated with different solvent extract of whole plant of *Lippia* and blood pressures were recorded every week during the entire period of the study by tail cuff method (Powerlab Non-Invasive Blood Pressure Instrument). At the end of the treatment, after a 12 hr of fasting and free access to deionised water, the animals were sacrificed. Blood samples were collected from retro-orbital plexus of rat. Each blood sample was allowed to clot and tubes were subsequently centrifuged at 2000 rpm for 5min to obtain sera which was transferred into new tubes and kept at -20˚ C until used for further analysis. Serum creatinine assay by alkaline picrate methods was carried out to check the serum creatinine level in animals and compared with the disease control group.

(VIII) Histopathological examination of renal tissues

Rats were sacrificed at the end of experiment and kidneys were isolated. Washed immediately with 0.9% saline and then fixed in 10% buffered neutral formaldehyde solution. Kidney was sliced, embedding in paraffin wax, sectioned (5-6 µ m) and stained with haematoxylin and eosin (H&E) dye. Slides were examined under a high power microscope (Nikon ELWD 0.3/OD75; Japan)[19].

STATISTICAL ANALYSIS

All results were reported as mean ± S.E.M. Data were analysed by one way analysis of Variance (ANOVA) followed by Tukey's test.

RESULTS

1. Preliminary qualitative phytochemical investigations

Preliminary phytochemical investigation of extracts of *Lippia nodiflora* revealed the presence of alkaloids, carbohydrates, flavonoids, saponins, tannins, triterpenoids and glycosides.

2. Evaluation of Anti-hypertensive activity

All four extract showed decrease in systolic blood pressure as shown in Table-2. Methanolic extract (500 mg/kg) significantly ($p < 0.001$) reduced blood pressure after 14 days of treatment as compared to disease control group (DOCA salt and 1%NaCl). Chloroform extract also showed significant reduction in blood pressure when compared to disease control group.

Table 2

Effects of different extracts of plant on systolic blood pressure (mmHg)

Group	0^{tn} day measurement of BP(mmHg)	7^{tn} day measurement of BP(mmHg)	14^{tn} day measurement of BP(mmHg)
Control	116.15±1.21	114.13±1.42	113.90±0.91
Sham control	116.70±0.95	114.26±1.74	116.36±0.75
Disease control	161.72±1.33#	161.10±1.27#	159.21±1.17#
Positive control	162.97±1.30	144.47±1.14*	120.1±0.98*
T1	164.03±0.67	150.67±1.18*	130.31±1.51*
T2	161.80±0.98	148.60±0.66*	134.51±0.94*
T3	165.40±0.81	153.75±0.77*	143.31±0.85*
T4	162.87±1.51	151.89±1.11*	146.24±0.92*

T1=treated with methanolic extract, T2=treated with chloroform extract, T3=treated with ethyl acetate, T4=treated with water extract. Each value expressed as mean ± S.E.M. Data were analysed by one way analysis of variance followed By Tukey's test (n=6).

 # p<0.001 when compared with control group using One way ANOVA followed by Tukey's Multiple range tests

**** p<0.001 when compared with disease control group using One way ANOVA followed by Tukey's multiple range tests.***

3. Estimation of serum creatinine

Serum creatinine levels of disease control group suggest renal impairment. There was significant improvement in serum creatinine levels when treated with methanolic extract of *Lippia nodiflora* (Table-3)

Table 3
Serum creatinine profile (mg/dl)

Groups	Serum creatinine 0th day(mg/dl)	Serum creatinine 14th day(mg/dl)
Control group	0.50 ±0.16	0.50 ±0.073
Sham control group	0.6 ±0.21	0.50 ± 0.044
Disease control	2.02 ± 0.16 #	2.36 ±0.089 #
Positive control	1.92 ± 0.15	1.87 ± 0.068
T1	2.05 ± 0.14	0.94 ±0.098 *
T2	2.10 ± 0.08	1.45 ± 0.095
T3	2.07 ± 0.06	2.01 ± 0.065
T4	2.03 ± 0.07	1.90 ± 0.054

T1=treated with methanolic extract, T2=treated with chloroform extract, T3=treated with ethyl acetate, T4=treated with water extract
Each value is expressed as mean ± S.E.M. (n=6)
 # p<0.01 when compared with normal control group using One way ANOVA followed by Tukey's Multiple range tests.
**** p<0.05 when compared with hypertensive group using One way ANOVA followed by Tukey's multiple range test***

4. Effect of extract on histopathology of kidney

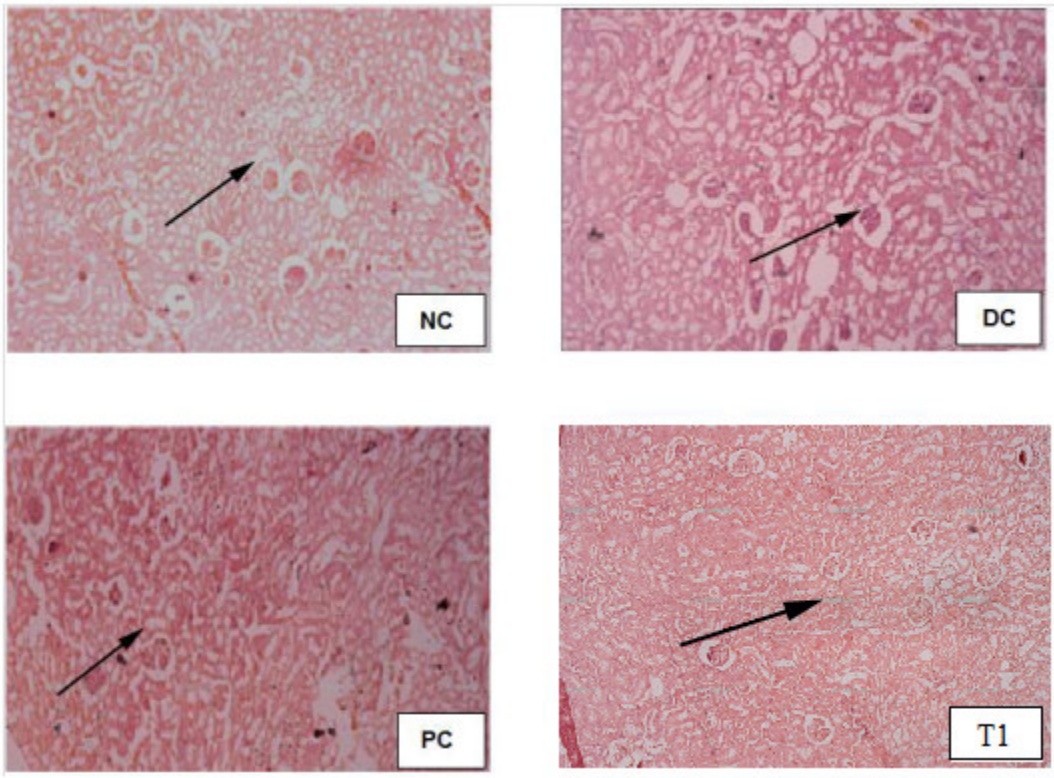

Figure 1
NC= Normal control group showing normal glomeruli and tubules, DC= Disease control group showing segmented and nephritic glomeruli. Tubules showing cloudy swelled parenchyma haemorrhage and inflammatory cells infiltrate in parenchyma. PC= positive control group showing mild swelling in tubules and few inflammatory cells. T1=Treated with methanolic extract group showing fewer inflammatory cells and near normal architecture.

DISCUSSION

Lippia nodiflora, possess antioxidant[20], and diuretic properties[21] may found to be useful for the treatment of hypertension. Preliminary study of Methanolic extract of this plant resulted in promising antihypertensive activity against DOCA salt induced hypertension in rats[22]. Selye *et al*[1957] were the first to demonstrate hypertensive property of DOCA. There is increased DOCA salt induced reabsorption of salt and water leading to

increased blood volume and ultimately blood pressure [23]. Unilateral nephroctomy stimulates peripheral RAAS and thereby sympathetic nervous system which accounts for this potential effect. Results of our study are in accordance with these previous findings showed significant increase in systolic blood pressure when compared with control animals[17.] Increased concentrations of aldosterone lead to increased reabsorption of sodium ions and water from epithelial cells in the distal nephron of the kidney, thereby influencing blood pressure levels [23]. In addition, increased aldosterone concentrations may activate oxidative stress through an unregulated NADPH oxidase in the DOCA-salt model [24]. Daily oral administration of our plant methanolic extract resulted in a remarkable reduction in systolic blood pressure. Flavonoids and triterpenes were recently shown to reduce hypertension in experimental animal models [25]. Several cohort studies have suggested that high intake of flavonoids may decrease the risk of coronary heart diseases [26]. The kidney plays a central role in the regulation of the balance of the body salt and water, and then disordered regulation of renal functions is responsible for the altered balance of salt and water in patho physiological states including some experimental models of hypertension [27]. Our results revealed that a considerable increase in plasma creatinine levels might indicate a hypertension in DOCA-salt treated rats and may be due to kidney damage caused by the oxidative stress by increasing the formation of superoxide. These finding correlate well with the kidney's histological examination. Oral administrations of our plant extract considerably decreasing the raised plasma creatinine level, bringing about remarkable recovery in kidney as evidence microscopically. This finding suggests that renal damage remarkably prevented by treatment with methanolic extract of plant in DOCA-salt hypertensive rats. It has been suggested that kidney injury can be minimized by reducing oxidative stress through increased intake of antioxidants [28]. Uninephrectomized-rat treated with methanolic fraction showed normal appearance of kidney without any pathological changes, and able to revert the damage occur in kidney tissues. This indicates that methanolic fraction of our plant possesses protective effect on kidney. Thus histopathological finding of the present study confirms the biochemical observation of this study.

CONCLUSION

In conclusion, methanol and chloroform extract have efficiently reduce systolic blood pressure in very short span of time. These extract produce anti-hypertensive effect by acting on multiple targets. The methanolic fractions showed the presence of flavonoids, Triterpenoids and it is reported that presence of flavonoids may show any anti oxidative effect on kidney may be reduced the stress that is produced by DOCA-salt on animals and helps in restoring the normal functions of kidney. Further investigation is projected be focused to undertake fractionation of these extract and find out individual constituent for above mentioned activity which will provide better and safer alternative treatment to control hypertensive conditions.

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