Phytochemical and pharmacological studies on methanolic seeds' extract of *Dolichos biflorus*

Mansoor Ahmad[1]*, Sadaf Sharif[1], Mehjabeen[2], Hina Sharif[1], Noor Jahan[3] and Ghazala Raza Naqvi[4]

[1]ResearchInstitute of Pharmaceutical Sciences, Department of Pharmacognosy, University of Karachi, Karachi, Pakistan
[2]Department of Pharmacology, Federal Urdu University of Arts, Science & Technology, Karachi, Pakistan
[3]Department of Pharmacology, Dow College of Pharmacy, Dow University of Health Sciences, Karachi, Pakistan
[4]Department of Pharmaceutics, Federal Urdu University of Arts Science and Technology, Karachi, Pakistan

Abstract: The *Dolichos biflorus* is a well known medicinal plant in folklore for its medicinal properties. In herbal medicine the seeds of it are mainly used as tonic, astringent, diuretic, and are also recommended in asthma, bronchitis, urinary discharges, hiccoughs, ozoena, heart trouble and other diseases of brain. The main purpose of this study is to explore and to provide experimental data on the traditional use of plant *Dolichos biflorus*. For this purpose we investigated the plant seed extract phytochemically and pharmacologically. Phytochemical analysis was performed on extract and powder form of the drug. Procedure use for evaluation were Identification of chemical constituent by color reaction, Fluorescence analysis of powder drug, pH (in powder and extract forms), loss on drying, Thin layer chromatography, Infrared spectroscopy, acid and saponification values. In pharmacological studies (diuretic, analgesic and anti-inflammatory activities) were tested on the extract of plant seed. The tests were carried out over albino mice taking different concentration of seed extract. Seeds extract of *Dolichos biflorus* has exhibited mild analgesic activity, the results were (84.6±6.68) at dose 300mg/kg and (92.2±6.81) at dose 500mg/kg which were not much significant as compared to reference drug Aspirin (300mg/kg) having result (36.4±2.27). While seed extract of *Dolichos biflorus* exhibited remarkable diuretic activity, the values at 300 mg/kg was (1.33±0.13) and at 500 mg/kg were (2.66±0.31) which are highly significant as compared to drug Lasix (20mg /kg) having result (2.38±0.23). Anti-inflammatory effects of crude extract of *Dolichos biflorus* obtained at 0.06mg/kg and 01mg/kg were (26.6±2.96) and (36±1.67) respectively. While the value for aspirin as standard drug (300mg/kg) were (17.44±1.59).This study provides a platform for further investigation for the isolation of active principles responsible for biological activity.

Keywords*: Dolichos biflorus*, Phytochemical, Analgesic, Antiinflammatory, Diuretic

INTRODUCTION

From the most ancient times, human being has had a primal belief that plants contain healing powers. Herbs have also been seen as a source of wisdom. In every part of the world, several medicinal plants are use for cure of specific ailments. Fortunately we have a huge number of plants grows wild in different parts of our country. Increased awareness about the useful plants has encouraged many new and progressive growers and entrepreneurs to take up their cultivation as a commercial enterprise. Apart from health care this enterprise provides means of livelihood to scores of people (Bhattacharjee, 1998). *Dolichos biflorus* is a well-known and wide spread genus of the family leguminoseae occurring mainly in the tropical countries. It contains most of the edible beans and many pulses. It is a climbing annual with slender pubescent stems (Bhattacharjee, 1998). It is a climbing annual herb with slender pubescent stems (Bhattacharjee, 1998).Leaves alternate and trifoliate having slender and hairy petiole; leaflets obliquely ovate, acute, entire margin, ciliate, pubescent, upto 31.7 cm (Anonymous, 2006). Flowers solitary or paired, axillary calyx campanulate, the upper two connate, other linear, hirsute, corolla of 5 pale-yellow petals, standard oblong, shortly clawed; fruit pod, recurved, sub globose or flat, beaked and straw brown (Anonymous,2006). The drug comprises of compressed seeds which are reniform, shinning, finely polished black or grey or brownish grey or reddish brown with purple and black spots; seed coat is very glistening, cotyledons two in number and whitish in color (Anonymous, 2006). Chemical constituents reported in the plants are streptogenin, β-sitosterol, bulbiformin, linoleic acid, polyphenols, oxalates and crude fiber. A number of isoflavones have been isolated from the leaves and stems namely genistein, dalbergioidin, kievitone, phaseollidin and isoferrerin (Keen *et al*., 1980).The seeds yield 5-hydroxy-7,3,4-trimethoxy-8-methylisoflavone-5-neohesperidoside, genistein, B-sitosterol and 5-o-α-L-rhamnopyranosyl (1-2)-β-D-glucopyranoside. Dolichin A and dolichin B have been obtained from bacteria treated leaves (Ingham *et al*., 1981). *Dolichos biflorus*, a commonly used legume in Uttarakhand, produces alpha amylase enzyme for conversion of starch present in its cotyledons to glucose (Garg and Dobriyal, 2011).

**Corresponding author:* e-mail:herbalist53@yahoo.com

Damodaran *et al*. (1948) suggested Arginine as the precursor of the amides (Narayana, 1930) studied amino acid and protein of plant *Dolichos biflorus.*

MATERIALS AND METHODS

Collection and identification of drug substance

The drug, seeds of *Dolichos biflorus* (Voucher specimen No.2008/DB/01SF) was collected from the local market of Karachi, Pakistan, and was identified by Prof. Dr. Mansoor Ahmad, Department of Pharmacognosy, University of Karachi, Pakistan).

Powdered drug preparation and extraction

Seeds of plant *Dolichos biflorus* were cleaned and crushed into fine powder by motor and pastle, then the powder material were sieved with the help of cotton cloth. For each sample separate piece of cloth was used to avoid contamination. For the extraction purpose the seeds of plant *Dolichos biflorus* macerated with ethanol for 15 days (2 times) at room temperature. The ethanol extract was then filtered and evaporated under reduced pressure in rotary evaporator with the water bath set at 40˚C to yield a residue.

PHYTOCHEMICAL EVALUATIONS

Color reaction

For the identification of different important constituent like carbohydrates, alkaloids, proteins, terpenes, tannins, saponins, simple qualitative tests were performed.

Powdered drug evaluation

Small quantity of powder was placed on a glass slide and one or two drops of iodine or glycerin or chloral hydrate solution was added. Later slide was observed under microscope and microscopic characters of each powder drug were studied. The presence of different tissues in the powder drug were identified and sketched with the help of pencil and distinct characters of the powder were recorded.

Thin layer chromatography

The following solvent systems were applied for TLC.
(i) Ethyl acetate-Methanol-Water (100:16.5:13.5).
(ii) Chloroform-Methanol-Water (80:20:2)

pH Determination (5mg/5ml)

1) In double distilled water
2) In distilled water
3) In sterile water
4) In plan water

Method

Place the electrode in the test solution and measure the pH at the same temperature as for the standard solution. At the end of a set of measurements, take a reading of the solution use to standardize the water and electrodes. If the difference between this reading and the original value is greater than 0.05, the set of measurement must be repeated. When measuring pH value above 10 make sure the glass electrode is suitable for use under alkaline condition and adjust as necessary. All solution of substance being examined and the reference buffer solution must be prepared using CO_2 free water (British Pharmacopoeia, 2004).

Acid value

The acid value is the number of mg of potassium hydroxide required to neutralize the free acid in 1 gram of the substance, when determined by the following method, unless otherwise stated in monograph.

Method

Weigh 10g of the substance (1 to 5g of resin or 1g of acid) into 250 ml flask, and add 50 ml of a mixture of equal volume of ethanol (96%) and ether which has been neutralized after the addition of 1 ml of dilute phenolphthalein solution .If necessary heat with caution until the substance is completely dissolved; titrate with 0.1ml KOH vs shaking constantly until a pink colour which persists for 15 sec is obtained. Note the number of ml required (v) calculate the acid value from the expression (British Pharmacopoeia, 2004).

Saponification value

The saponification value is the number of mg of potassium hydroxide required to neutralize the fatty acid from 1gm of the substance.

Method

Dissolve 40gm of KOH in 20ml of water and add sufficient 96% ethanol to produce 1000ml. Allow to stand overnight, and pour off the clear liquid. Weigh 2 gm of the substance in a 200ml flask and 25ml of the ethanol solution of potassium hydroxide, attach a reflux condenser and boil for 1 hour, frequently rotating the content, while the solution is still hot and 1ml of phenolphathalin solution and titrate the excess of alkali with 0.5m HCl vs. note the number of milliliters required (British Pharmacopoeia, 2004).

Loss on drying

Loss on drying is the loss of weight expressed as % w/w.

Method

Place the prescribed quantity of the substance to be examined in a weighing bottle previously dried under the conditions prescribed for the substance to be examined. Dry the substance to constant mass or for the prescribed time by one of the following procedures. Where the drying temperature is indicated by a single value rather than a range, drying is carried out at the prescribed temperature ± 2°C (British Pharmacopoeia, 2004; Ph. Eur. 2005).

Infrared spectro-photometry

Infrared spectrophotometers are used for recording spectra in the region of 4000-650 cm-1 (2.5-15.4 μm) or in some cases down to 200 cm^{-1} (50 μm) (British Pharmacopoeia, 2004). The absorbance (A) is defined as the logarithm to base 10 of the reciprocal of the transmittance (T):

A= log10 (I/T) = log10 (Io/I)

T= I/ Io, Io = intensity of incident radiation, I = intensity of transmitted radiation.

PHARMACOLOGICAL STUDIES

Experimental animals

Albino mice of either sex obtained from H.E.J. Research Institute of Chemistry, Karachi, Pakistan were used to determine the analgesic, diuretic, and CNS activities. Mice weighing 25-30 gm. Animals were kept in colony cages (five animals in each group) with access to food and water. They were maintained in a climate and light controlled room (30°C±1ºC 12/12 hours light/dark cycle) at least 7 days before testing or administration of the drug. The drug Diazepam in concentration of 300mg and 500mg were used for experimental purpose.

Diuretic activity of Dolichos biflorus

The diuretic effect of methanol extracts of the dried seeds of *Dolichos biflorus* in normal micewas evaluated by the method of Umang *et al*. (2009). Aqueous and methanol extracts of *Dolichos biflorus*seeds were administered to experimental mice orally at doses of 300mg/kg and 500 mg/kg . Hydrochlorothiazide (10 mg/kg) was used as positive control in study. The diuretic effect of the extracts was evaluated by measuring urine volume in ml (Umang *et al*. 2009).

Anti inflammatory activity of Dolichos biflorus

The Formalin induced inflammation was monitored by licking and biting and mean paw. The results show decrease in inflammation of hind paw of drug-treated animals as compared to the control group. This finding suggests that the crude extract of *Dolichos biflorus p*ossesses mild anti-inflammatory activity. The crude extract was tested in two doses as 300 mg/kg and 500 mg/kg of test animals. It was found that the test substance at 500 mg/kg is more effective than 300 mg/kg. The result of drug treated animals when compared with standard (Aspirin, 300mg/kg) manifested that anti-inflammatory activity of crude extract of *Dolichos biflorus* less significant than standard drug.

Analgesic activity of Dolichos biflorus

These tests were performed according to the modified method of Koster *et al* (1959) and Turner (1965). Mice were used as the test animals in this method. According to this method writhes were induced by intraperitonial administration of the acetic acid solution 10ml/kg thirty minutes prior to the administration of the acetic acid, the animals were treated orally with the test substance. Numbers of writhes was counted for 30 minutes immediately after acetic acid administration. A reduction in the number of writhing as compared to the control animals was considered as evidence for the presence of analgesia and expressed as percent.

STATISTICAL ANALYSIS

The data are expressed as mean standard error means (SEM) using one-way ANOVA. Values at $p<0.01$ were considered as statistically significant (*).

RESULT

Phytochemical analysis and standerdization

Preliminary phytochemical screening is reported in table 1. In the phytochemical screening of methanolic extract of plant seed compounds like reducing sugar, tannins are found to be present. Phenolic compounds, saponin and protein are also present. Powder drug evaluation reveled the presence of Epidermal cells, Epidermal cells with radical, Parenchymatous cells of radical, parenchymatous cells of cotyledon, hypodermal cells and starch grain**.** Fluorescence analysis of powder drug presented in table 2. In which powder of *Dolichos biflorus* treated with different chemicals and visualized under ordinary and UV light (254nm and 366nm).

pH determination

The pH of powder drug and extract (0.1gm/5ml) of *Dolichos biflorus* were evaluated using different form of water. The results demonstrated in table 3 exhibit the acidic nature of the plant extract and powder.

Loss on drying

The drying of powdered drug was carried out in an oven within the temperature range 105°C and recorded as 5.35± 0.16.

Acid value

The acid value of crude methanolic extract of *Dolichos biflorus* seed was found to be 244.24±0.28.

Saponification value

Crude methanolic extract of seed of *Dolichos biflorus* resulted in mean value as 12.025± 0.29.

Infrared spectrophotometry

Infrared spectrophotometric study of powder drug resulted in following wave numbers. 2925.31 cm^{-1} (Aliphatic C-H), 1744.38 cm^{-1} (C=O), 1609 cm^{-1} (C=O) stretch carbonyls, 15O7.38 cm^{-1} (N–O), 1405.22 cm^{-1} (Benzene), 1192.74 (C–N) stretch aliphatic amines, 927.13 cm^{-1} O–H bend carboxylic acids, 861.75 cm^{-1}C–H "oop" aromatics, 759.59 cm^{-1} (C–Cl) stretch alkyl halides.

Table 1: Florescence analysis of powder drug *Dolichosbiflorus*

S. No.	Protocols	Ordinary Light	UV LIGHT 254nm	UV light 366nmite
1	Dry powder	Cream color	White	Off white
2	Powder and NaOH in MeOH	Brownish green	Light green	Off white
3	Powder treated with 1.0N HCL	Off white	White	White
4	Powder treated with 1.0N NaOH in H_2O	Dark brown	Green	Dark brown
5	Powder treated with 50%HNO_3 aqueous	Greenish brown	Green	Brown
6	Powder treated with 50% H_2SO_4 aqueous	Brown	Green	Dark brown
7	Glacial acetic acid	Off white	Off white	Dark brown
8	Ethanol	No change	Light green	Dark brown
9	Acetone	Off white	No change	Brown
10	Methanol	No change	Light green	Brown color
11	Iodine solution	Dark green	Off white	Dark brown

Infrared spectrophotometric study of extract resulted in following wave numbers. 3002.05 cm^{-1} (aromatic C-H),2925.31 cm^{-1} (Aliphatic C-H), 1732.12 cm^{-1} (C=O), 1642.22cm^{-1} and 1580 cm^{-1} (Benzene) and 1540.07cm^{-1} (C-O-C), 1245.86 cm^{-1} (C-N), 1151 cm^{-1} (C-O Alcohols), 869.92 cm^{-1} (C-H alkene), 808.63 (C-Cl stretch alkyl halides).

Diuretic activity

Diuretics play an important role in situations of fluid overload like acute renal failure hypercalciurea, Cirrhosis of liver. They decrease plasma volume and subsequently venous return to the heart. This decreases cardiac workload, oxygen demand and plasma volume thus decreasing blood pressure, therefore diuretics plays an important role in antihypertensive patients. Data obtain from the *Dolichos biflorus* diuretic studies indicate that its extract produced significant and dose dependent diuretic activity. The values for control step are 0.95±0.07, while when 300mg concentration of drug is used the values are 1.33±0.13, for 500mg the values are 2.66±0.31. The values for standard drug are 2.38± 0.23.

Analgesic activity

Following experiment is performed in order to determine the analgesic activity of plant *Dolichos biflorus*. For that purpose acetic acid solution was subjected to mice. The procedure is explained in experimental. The controls values obtain are 108±3.25, values for 300mg concentration is 84.6±6.68 and for 500mg concentration the values obtain are 92.2±6.81.

Anti-inflammatory activity

In order to determine the anti-inflammatory effect of crude extract of *Dolichos biflorus* following test is performed. From the result it can easily be conclude that the plant have mild anti-inflammatory activity. The results values for control observation are 69±5.46. For 300mg and 500mg the values are 26.6±2.96 and 36±1.67 respectively. While the value for Aspirin as standard drug is 17.44±1.59.

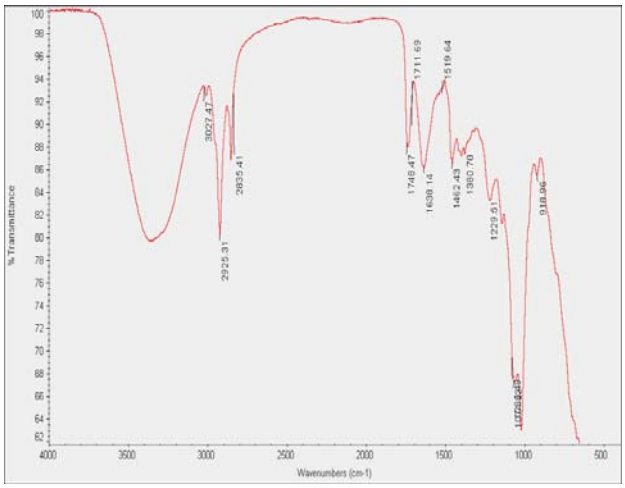


Fig. 1: FTIR of *Dolichos biflorus* in powder form.

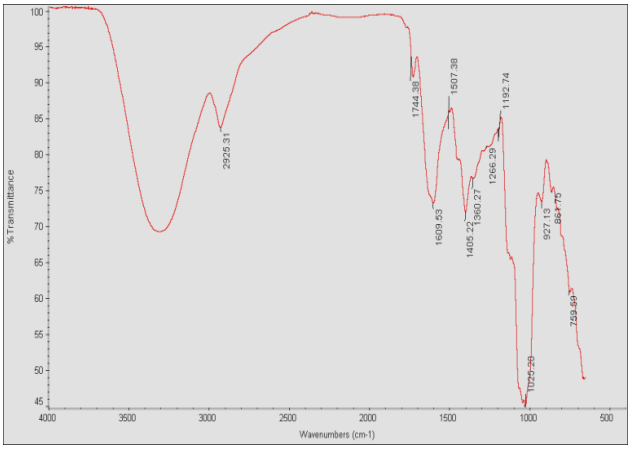
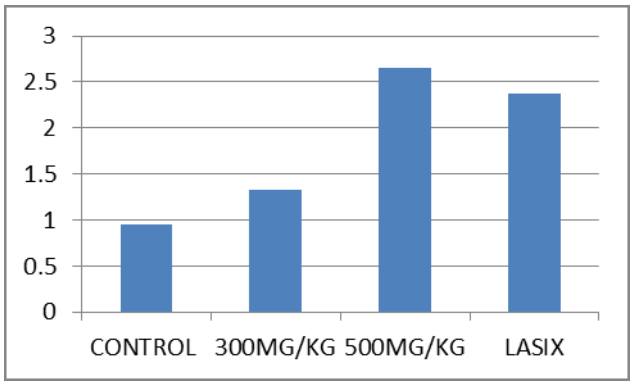


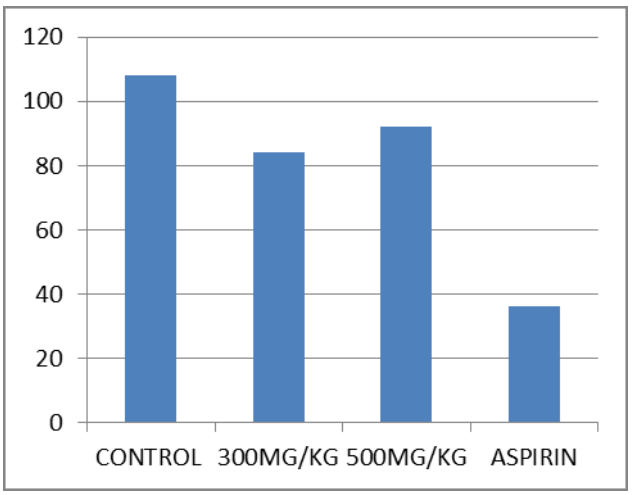
Fig. 2: FTIR of *Dolichos biflorus* extract form.

Table 2: Phytochemical screening and physical characteristic of *Dolichos biflorus*

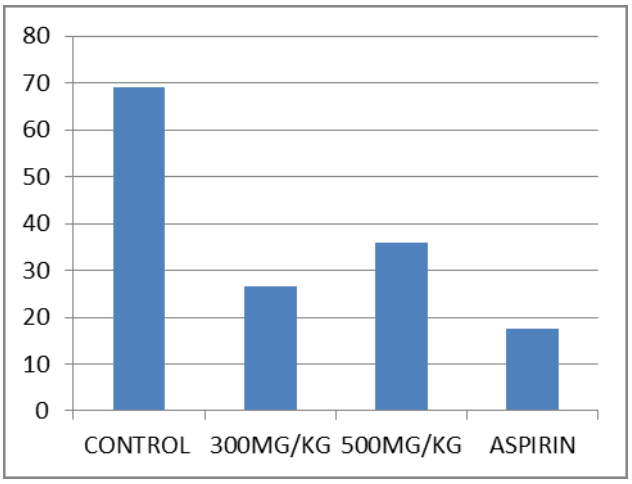
Physical characteristic	Values
Saponification value	12.025± 0.29
LOD	5.35±0.16
Acid value	244.24± 0.28
TLC (R_f)	0.35 & (0.16 & 0.31)
pH (powder & extract)	
Plane water	6.67±0.20 & 6.856± 0.14
Distill water	6.13±0.21 & 5.228±0.09
Sterile water	6.19±40.15 & 5.294±0.16
D. Distill water	6.16±0.07 & 5.63±0.26
Phytochemicals	Methanolic Extract
Triterpenes	+
Tannins	+
Carbohydrates	+
Alkaloids	-
Proteins	+
Sterols	+
Saponin	+



Graph 1: Diuretic activity of *Dolichos biflorus*



Graph 2: Analgesic activity of *Dolichos biflorus*



Graph 3: Antiinflammatory activity of *Dolichos biflorus*

Table 3: FTIR values of *Dolichos biflorus*

Dolichos biflorus in powder form			*Dolichos biflorus* in extract form	
S. No.	Wave Number (cm-1)	Functional group	Wave Number (cm-1)	Functional group
1	2925.31	Alkyl C-H Stretch	3002.05	Alkenyl C-H, Alkenyl C=C Stretch
2	1744.38	C=O stretch esters, saturated aliphatic	2925.31	Alkyl C-H Stretch
3	1609.53	C=O stretch carbonyls (general	1732.12	C=O stretch aldehydes, saturated aliphatic
4	1507.38	N–O asymmetric stretch(nitro comp)	1642.22	C=C– stretch alkenes
5	1405.22	C–C stretch (in–ring) aromatics	1580.93	C–C stretch (in–ring) aromatics
6	1360.27	N–O asymmetric stretch(nitro comp	1540.07	N–O asymmetric stretch(nitro comp)
7	1192.74	C–N stretch aliphatic amines	1245.86	C–N stretch aromatic amines
8	927.13	O–H bend carboxylic acids	1151,87	C–O stretch alcohols, carboxylic acids, esters, ethers
9	861.75	C–H "oop" aromatics	914.87	O–H bend carboxylic acids
10	759.59	C–Cl stretch alkyl halides	869.92	C–H "oop" aromatics
11			874.01	=C–H bend alkenes
12			869.92	N–H wag primary, secondary amines
13			808.63	C–Cl stretch alkyl halides

Table 4: Diuretic activity of *Dolichos biflorus*

Treatment	Dose (mg/kg)	Mean No. of ml
Control	0.5ml saline	0.95 ± 0.07
DB	300	1.33± 0.13
-	500	2.66 ± 0.31
Lasix	20	2.38 ± 0.23

Each value is the mean ± S.E.M. of five determinations.
**P* < 0.05, Dunnet test as compared to control.

Table 5: Analgesic activity of *Dolichos biflorus*

Treatment	Dose (mg/kg)	Mean No. of writhes
Control	0.5ml saline	108± 3.25
DB	300	84±6.68
-	500	92.2 ± 6.81
Aspirin	300	36.4 ± 2.27

Each value is the mean ± S.E.M. of five determinations.
**P* < 0.05, Dunnet test as compared to control

Table 6: Antiinflammatory activity of *Dolichos biflorus*

Treatment	Dose (mg/kg)	Mean No. of ml
Control	0.5ml saline	69± 5.46
DB	300	26.6 ± 2.96
-	500	36 ± 1.67
Aspirin	300	17.44 ± 1.59

Each value is the mean ± S.E.M. of five determinations.
* *P* < 0.05, Dunnet test as compared to control.

DISCUSSION

The following research work was carried out on medicinal plant *Dolichos biflorus*. The aim and objective of this research was to find out the hidden properties of it, which are yet to be found helpful for mankind in numbers of ways. During research work the major emphasis was given over the parameters, which are important in utilization of this plant in medicine. Nowadays intensive utilization of herbal medicine all over the world creates some problems to human bodies (Bhattacharjee, 1998). Therefore, different countries made their rules and regulations for import and export, manufacturing and selling of herbal drugs. With the same ideas this research work is also carried out. Standardization of herbal drugs and extract is an essential part and necessary for establishment of medicines and their pre clinical and clinical trials. Phytochemical study determines the purity of drugs and indicates the presence or absence of active constituents. Therefore, to accomplish equivalent response of therapy; one has to be familiar with the standardized methods of drugs that was used in the study.

Phytochemical and pharmacological studies

The crude extract of *Dolichos biflorus* was analyzed chemically with various chemical reagents. For the detection of various chemical classes color reaction test was performed, positive result for CHO, protein, saponin and sterols obtained. For the standardization of crude extract of *Dolichos biflorus* following tests were carry out: Acid value, Saponification value, pH and FTIR tests. The FTIR results of *Dolichos biflorus* in extract and in powder form showed the presence of different functional groups like alkyl, saturated aliphatic, carbonyl, aromatic, alcohols ether and esters groups.The pH of *Dolichos biflorus* extract and powder form determine by using plane water, Distill water, Sterile water and Double distill water. Through the result it can be conclude that the plant rather it is in powder or in extract form is acidic in nature.

Diuretic activity

Plant *Dolichos biflorus* is famous for its diuretic property in folklore. Through results we concluded that plant has excellent diuretic property. Plant extract in different dose concentration i.e. 300mg/kg and 500mg/kg were used on mice. Maximum results were obtained at high doses i.e. 500mg in comparison to reference drug, Lasix (Umang *et al*. 2009).

Analgesic activity

From the result it can be concluded that the plant *Dolichos biflorus* exhibited mild analgesic activity. Plant seed extract in concentration of 300mg/kg and 500mg/kg is subjected to different groups of mice. No pronounced analgesic activity was observed in either dose. The results were less significant as compared to control and standard drug. (Koster *et al.,*1959). The mild analgesic activity of metanolic extract of *Dolichos biflorus* is due to the presence of active principle like triterpenoids, reducing sugar, phenolic compounds, saponin, xanthoprotein, tannin, flavonoids and aromatic acids.

Anti-inflammatory activity

Dolichos biflorus seed's extract in concentration of 300mg/kg and 500mg/kg doses were introduced to mice for anti-inflammatory effects. Mild effects were observed. Mild anti-inflammatory effects are noticed that were not significant as compared to control and standard drug Aspirin. The active principles responsible for anti-inflammatory activity are reducing sugar, saponin, tannin, flavonoids and aromatic acid.

CONCLUSION

In search of novel plant the study lead us to the point where we can conclude that the plant *Dolichos biflorus* is a rich source of many bioactive compounds. The presence of these compounds makes this plant as a potential diuretic plant. It has mild analgesic and anti-inflammatory activity. This study does not reveal the active compound

that's responsible for the diuretic activity. Now we will direct our investigation to figure out the main compound from this plant responsible for diuretic activity.

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