International Standard Serial Number (ISSN): 2249-6807

International Journal of Institutional Pharmacy and Life Sciences 2(3): May-June 2012

INTERNATIONAL JOURNAL OF INSTITUTIONAL PHARMACY AND LIFE SCIENCES

Pharmaceutical Sciences **Research Article……!!!**

Received: 27-05-2012; Revised; Accepted: 29-05-2012

ANALGESIC ACTIVITY OF HYDRO-ALCOHOLIC EXTRACT OF *OCIMUM CANUM* IN WISTAR ALBINO RATS

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Keywords:

Ocimum canum, hydro-alcoholic, antioxidants, analgesic

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ABSTRACT

Antioxidant activity of the hydro-alcoholic extract of *Ocimum canum* Sims (Hairy Basil) was determined by various antioxidant assays. In all the testing, a significant correlation existed between concentrations of the extract and percentage inhibition of free radicals. The analgesic activity of phyto-constituent from the leaf of *Ocimum canum* was studied after oral administration in rats using the tail flick, hot plate and acetic acid-induced writhing methods. The results were compared with aspirin. It was found that *O. canum* increased the tail withdrawal latency in rats. The extract was found to have a significant inhibitory effect on hotplate method at all the doses. Using the acetic acid-induced writhing method, the extract showed significant inhibition in a dose-dependent manner suggesting its possible mechanism related to the peripheral system. The results of our study suggest that the analgesic action of OC is exerted both centrally as well as peripherally and involves interplay between various neurotransmitter systems. Preliminary phytochemical screening revealed the presence of alkaloids, saponins, tannins and flavonoids. The results suggest the extract contained pharmacologically active principles, and supports the local application of the plant in painful conditions. Further studies may reveal the exact mechanisms of action responsible for the analgesic activities of *O. canum* leaves extract.

INTRODUCTION

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage. Excessive pain may be unbearable and cause other effects like sinking sensation, sweating, nausea, palpitation, rise or fall in B.P, tachycardia. It is useful to distinguish two basic types of .pain, acute and chronic, and they differ greatly. Acute pain for the most part, results from disease, inflammation, or injury to tissues. This type of generally comes on suddenly, for example, after trauma or surgery, and may be accompanied by anxiety or emotional distress. The cause of acute pain can usually be diagnosed and treated, and the pain is self-limited, that is, it is confined to a given period of time and severity. In some rare instances, it can become chronic. Chronic pain is widely believed to represent disease itself. It can be made much worse by environmental and psychological factors. Chronic pain persists over a longer period of time than acute pain and resistant to most medical treatment. It can often does cause severe problems for patients.

Ocimum canum (Ram tulsi or Kali tulsi) traditionally employed intensively as folklore remedy for a wide spectrum of diseases in India (P. Prakash and Neelu Gupta, 2005). Every part of *Ocimum canum* is reported to be associated with various remedial properties such as, antimicrobial effects (J. J. C. Scheffer*et al*., 2002)

***Fig.: Ocimum canum* Sims (Hairy Basil) Wild basil, from Yamasaki Lab. Plant Photo Gallery, by kind permission from Kazuo Yamasaki.**

MATERIALS AND METHODS

Chemicals used:

Sl no.	Chemicals name	Suppliers
1	Hydro-alcoholic extract of *O. canum*	Self-prepared in Pharmacology lab of RCPHS
2	Distilled water	Pharmaceutics lab of RCPHS
3	Ascorbic acid	Merk Laboratory
4	Hydrogen peroxide	Merk Laboratory
5	DPPH	LobaChem
6	H_2SO_4	LobaChem
7	Potassium Iodide	LobaChem
8	Mercuric chloride	LobaChem
9	Bismuth carbonate	LobaChem
10	Glacial acetic acid	LobaChem
11	Acetic acid	LobaChem
12	Aqueous picric acid	LobaChem
13	Benedict's Reagent	LobaChem
14	Fehling's solution	LobaChem
15	Ferric chloride	LobaChem
16	Pyridine	LobaChem
17	Lead acetate	LobaChem
18	Sodium chloride	LobaChem
19	NaOH	LobaChem
20	Ninhydrine solution	LobaChem
21	HNO_3	LobaChem
22	$NaNO_2$	LobaChem
23	α – napthol	LobaChem
24	Chloroform	LobaChem
25	Acetic anhydride	LobaChem
26	Aspirin	LobaChem
27	Lenoleic acid	LobaChem

Animals:

Adult rats of either sex (150-200gm) were obtained from the animal house of R.C.P.H.S. and were housed and divided into 4 groups containing 8 animals each. All the experimental procedures and protocols used in this study were reviewed and approved by Institutional Animal Ethical Committee, Regd. No: - 07/IAEC/2011.

Preparation of plant extract:

The leaves of *Ocimum canum* were dried for 20 days under the shade to prevent the loss of volatile oils. The shade-dried and powdered leaves were subjected to extraction with hydro-alcoholic extract by maceration .The hydro-alcoholic mixture was prepared by ethanol 70% and water in the ratio of 7:3. The filtrate was collected and concentrated on heating mantle to obtain a syrupy mass. The extracts were subjected to phytochemical study as well as pharmacological activity.

Administration of the extracts:

Suspension of hydro-alcoholic extract was prepared in distilled water. The extract was administered in a dose of 200 & 400 mg/kg to rats by oral route, 45 min before the test procedures for pre-pharmacological screening as per OECD guidelines. Control group was given only the vehicle (distilled water) in volume equivalent to that of plant extract.

Phytochemical screening:

A) Test for Alkaloids

1 **Wagner's Reagent Test:** With alkaloid it shows reddish brown precipitate. It was prepared by dissolving 1.27 gm of Iodine and 2 gm of Potassium Iodide in 5 ml of water & the final volume was mace up to 200 ml.

2 **Mayer's Reagent Test**: To prepare this reagent, 1.36 gm of mercuric chloride was dissolved in distilled water. In another part dissolve 5 gm of potassium iodide in 60 ml of distilled water then both the part were mixed and the volume was adjusted to 200 ml. With alkaloids it shows white to buff precipitate.

3 **Dragendorff's Reagent Test**: With alkaloids this reagent gives orange-brown coloured precipitate. To prepare this reagent, 14 gm of sodium iodide was boiled with 5.2 gm of bismuth carbonate in 50 ml glacial acetic acid for few minutes. Then it was allowed to stand for overnight and the precipitate of sodium acetate was filtered out. To 40 ml of filtrate 160 ml of acetate and 1 ml of water was added. The stock solution was stored in amber coloured bottle. During experiment, 10 ml of stock solution and 20 ml of acetic acid was added and the final volume was made up to 100 ml with water.

B) Test for Carbohydrate

1 **Benedict 's Test**: In this method of test for monosaccharide, 5 ml of Benedict's reagent and 3 ml of test solution when boiled on a water bath a brick red precipitate appears at bottom of the test tube confirms the presence of the compound.

2 **Fehling's Test:** In this method 2 ml of Fehling 'A', 2ml of Fehling 'B' and 2 ml of extract were boiled. The presence of reducing sugar is confirmed if yellow or brick red precipitate appears at the bottom of the test tube confirms the presence of the monosaccharide.

3 **Molisch's Test:** When the alcoholic solution of the extract and 10% alcoholic solution of α-napthol were shaken and concentrated sulphuric acid was added along the side of the test tube, a violet ring at the junction of two liquids confirms presence of carbohydrates.

C) Test for Glycosides

1 **Keller-killiani Test:** To a hydro-alcoholic extract of the drug in glacial acetic acid few drops of ferric chloride and concentrated sulphuric acid was added. A reddish brown colour is formed at the junction of the two layers and upper layer turns bluish green.

2 **Legal Test:** To a solution of glycoside in pyridine, sodium nitropruside solution and sodium hydroxide solution were added. A pink to red colour will confirm the presence of glycosides.

D) Test for Tannins and Phenolic Compounds

1 **Test with Lead Acetate:** Tannins get precipitate with lead acetate

2 **Test with Ferric Chloride**: Generally phenols were precipitate with 5% w/v solution of ferric chloride in 90% alcohol and thus phenols are detected.

3 **Test with Gelatin solution**: To a solution of tannins (1%), aqueous of gelatin (1%) and sodium chloride (10%) were added. A white buff precipitate confirms the compound.

E) Test for Proteins and Amino acid

1 **Biuret Test:** When 2 ml of the hydro-alcoholic extract, 2ml of 10% sodium hydroxide solution and 2-3 drops of 1% copper sulphate solution were mixed, the appearance of violet or purple colour confirms the presence of proteins

2 **Ninhydrin Test:** When 0.5 ml of ninhydrin solution is added to 2 ml of the hydro-alcoholic extract and boiled for 2 minute and then cooled. The appearance of blue colour confirms the presence of proteins.

F) Test for Gums and Mucilage's

Ruthenium Red Test: In this test 0.08gm of ruthenium red when dissolved in 10 ml of 10% solution of lead acetate. It stains to red colour.

Molisch's Test: when the hydro-alcoholic extract and 10% alcoholic solution of α-naphthol were shaken and concentrated Sulphuric acid was added along the side of the test tube, a violet ring at the junction of two liquids confirms presence of carbohydrate, gums and mucilage.

Test with 95% Alcohol: When 95% alcohol added to the hydro-alcoholic extract, gums get precipitated out. The precipitate is insoluble in alcohol.

G) Test for Flavonoids

Test with sodium hydroxide (NaOH)

For the detection of flavonoids the hydro-alcoholic extract was dissolved with water.It was filtered and the filtrate was treated with NaOH a yellow coloured confirms the presence of flavonoids.

Test with sulphuric acid

A drop of H_2SO_4 when added to above, the yellow colour disappears.

H) Test for saponins

Foam Test

About 1ml of hydro-alcoholic extract was diluted separately with distilled water to make the volume up to 10 ml and shaken in graduated cylinder for 15 minutes indicates the presence of saponins.

I) Test for steroids and sterols

Salkowski's Test:

To 5ml of hydro-alcoholic extract in chloroform in dry test tube equal volume of conc. H_2SO_4 was added alongside of the test tube. The presence of sterols & steroids are confirmed by upper layer of chloroform showing a play of colours first bluish red to gradually violet and lower acid layer showing yellowcolour with green fluorescence.

LibermannBurchard Reagent Test:

In this method of detection, about 2ml of solution of hydro-alcoholic extract was dissolved in chloroform was placed in dry test tube. Then 2ml of acetic anhydride and 2-3 drops of conc. H_2SO_4 was added to it and allowed to stand for few minutes. An emerald green colour develops if steroid or sterols are present

J) Test for Terpinoids:

Test with Tin &Thionyl Chloride:

For detection of teriterpinoids the hydro-alcoholic extract was dissolved in chloroform. A piece of metallic tin and 1drop of thionyl chloride was added to it .Pink colour confirms the results.

Employing *in vitro* model:

a) DPPH radical scavenging assay:

To the Ethanol solution of DPPH (1 mM) an equal volume of the extract dissolved in alcohol was added at various concentrations from 250 to 2000 µg/ml in a final volume of 1.0 ml. An equal amount of alcohol was added to the control. After 20 min, absorbance was recorded at 517 nm Experiment was performed in triplicate

b) Hydrogen peroxide radical scavenging study:

The scavenging of hydrogen peroxide by the standard (ascorbic acid) and extract after incubation for 10 minutes increased with increased concentration. *Ocimum canum* hydro-alcoholic extracts exhibited higher hydrogen peroxide scavenging activity than ascorbic acids at similar concentrations. While hydrogen peroxide itself is not very reactive, it can generate the highly reactive hydroxyl radical (OH) through the Fenton reaction *(Equation* 1) Thus, the scavenging of hydrogen peroxide is an important antioxidant defense mechanism.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^- \textit{(Equation 1)}$$

The decomposition of hydrogen peroxide to water involves the transfer of electrons as in Equation 2.

$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O \quad \textit{(Equation 2)}$$

The scavenging of hydrogen peroxide by phenolic compounds has been attributed to their electron-donating ability. The hydro-alcoholic OC extracts have high electron-donating abilities, and 68.21 ± 0.35scavenging was achieved with concentrations of hydro-alcoholic extracts at 2000 µg/ml.In comparison, the hydrogen peroxide scavenging activity of ascorbic acid at 2000 µg/ml were found to be 98.79 ± 0.28.

Employing *in vivo* model:

Hot plate method:

Analgesic activity was tested in rats using the hot plate method of Janssen and Jagneau (1957). Eight rats were divided in to four groups. One group was given only distilled water and observed as a control. The second group was given Aspirin and treated as

standard and other group was given test drugs i.e. HAOC 200 & 400 mg/kg. Swiss rats were placed in aluminum hot plate kept at a temperature of 55 ± 0.5 degree centigrade for a maximum time of 15 seconds. Reaction time was recorded when animals licked their paws or jumped. The responses were taken at different time interval i.e. 0,30,60,90,120,180 & 240 minutes after oral administration of hydro-alcoholic extract with dose of 200 and 400 mg/kg respectively. Cut off time in the absence of a response was 15 sec to prevent the animals from being burnt (Sharma *et al.,* 1982).

Tail flick method:

Total of eight rats divided in the groups of three each, and three groups were made .One group was given only distilled water and observed as a control. The second group was given Aspirin and treated as standard and other group was given test drugs i.e. HAOC 200 & 400 mg/kg Aspirin was taken as standard drug. All the drugs were given intraperitonially. The tail flick latency was assessed by analgesiometer (INCO, INDIA).The strength of the current passing through the naked nichrome wire was kept constant at 6 amperes. The distance between the heat source and tail skin was 1. 5 cm. The site of application of the radiant heat in the tail was maintained at2.5c.m.measured from the root of tail. The cutoff reaction time was fixed at 10 seconds to avoid tissue damage.

Acetic acid writhing test:

Anti noociceptive response of the extract *Ocimum canum* (200 and 400 mg/kg) was assessed by counting number of writhes (constriction of abdomen, turning of trunk and extension of hind legs) induced by 1% acetic acid solution (1mL:100 g) in rats. Number of writhes per animal was counted during 30 min test period, beginning 3 min after the injection of acetic acid. Acetyl salicylic acid 100 mg/kg body weight was used as a reference drug. Total of 8 rats divided in four groups .One group was given only distilled water and observed as a control. The second group was given Aspirin and treated as standard. Other groups were given test drugs i.e.HAOC 200 & 400 mg/kg.

RESULTS

Phytochemical Screening:

TABLE 1:- Result of Phytochemical Study

SL NO.	PHYTOCONSTITUENTS	HYDRO-ALCOHOLIC EXTRACT	AQUEOUS
	Alkaloid	+ + + -	+ + + -
2	Carbohydrate	- - +	- - +
3	Glycoside	++	+ +
4	Tannins	+ - -	+ +
5	Protein & Amino acid	_ _ _	- - -
6	Gum and Mucilage	---	+ + +
7	Flavones & Flavonoids	++	+ +
8	Saponins	+	+
9	Steroids & Sterols	+	+
10	Triterpenoids	+	+

+ indicates presence, - indicates absence

In vitro evaluation:

a) DPPH radical scavenging assay:

TABLE 2:-Study on DPPH scavenging activity in *Ocimum canum*

Concentration (µg/ml)	Ascorbic acid (% scavenging activity)	*O. canum*(% scavenging activity)
0	0	0
250	90.2±0.004	46.8 ± 0.005
500	91 ±0.009	56.7 ± 0.003
1000	92.4 ±0.005	72 ± 0.005
2000	93±0.007	79.2 ± 0.004

Statistical analysis: Data are expressed as mean ± SEM (standard error of mean) for five animals. The difference among means has been analyzed by student- t test.

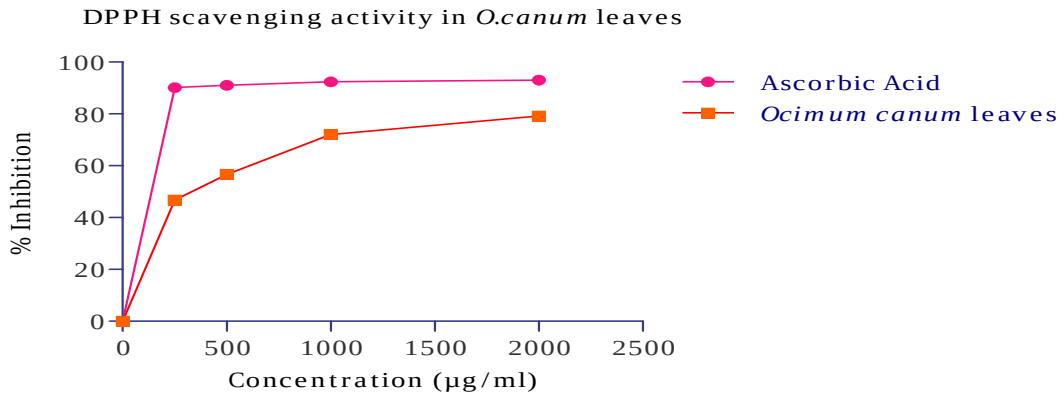

FIGURE 1:-Study on DPPH scavenging activity in *Ocimum canum*leaves at 517 nm

b) Hydrogen peroxide radical scavenging study:

TABLE 3:-Scavenging Activity of Hydrogen Peroxide in *Ocimum canum*

Concentration (µg/ml)	Ascorbic acid (% scavenging activity)	*O.canum* (% scavenging activity)
0	0	0
250	52.74 ± 0.22	30.70 ± 0.23
500	62.63 ± 0.34	41.27 ± 0.38
1000	78.57 ± 0.32	58.54 ± 0.28
2000	98.79 ± 0.28	68.21 ± 0.35

Statistical analysis: Data are expressed as mean ± SEM (standard error of mean) for five animals. The difference among means has been analyzed by student- t test.

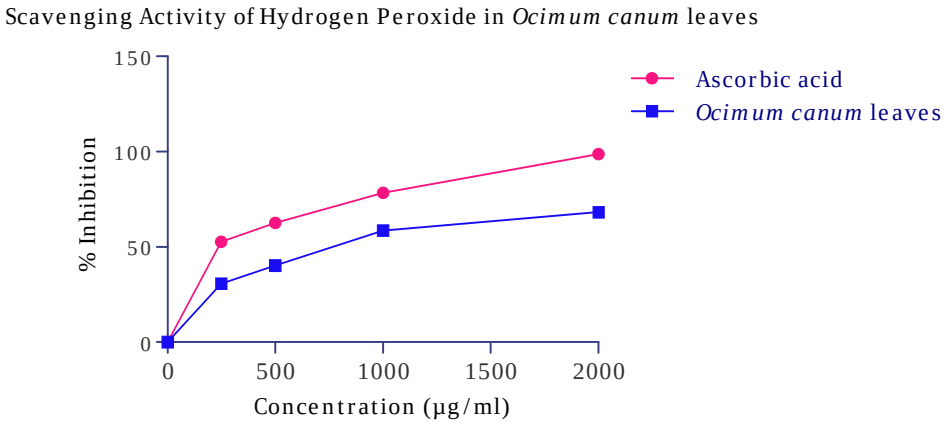

FIGURE 2:-Study on Scavenging Activity of Hydrogen Peroxide in *Ocimum canum* leaves at 560 nm

***In vivo* evaluation: Hot Plate Method:**

TABLE4:- Mean Response of Hot Plate method at Various Time Interval

Treatment	Dose (mg/kg)	Mean Basal Time (Seconds)	Mean Response (in seconds) at Various Time Interval (in minutes)					
			30	60	90	120	180	240
Distilled water	0.5ml	4.39± 0.019	4.16± 0.186	4.69± 0.028	4.78± 0.041	4.19± 0.035	4.07± 0.047	4.55± 0.035
Aspirin	10	4.45± 0.037	7.68± 0.042	8.61± 0.025	9.47± 0.047	11.18± 0.038	10.68± 0.038	9.95± 0.041
HAOC	200	4.63± 0.037	4.87± 0.031	5.19± 0.035	5.26± 0.044	5.94± 0.046	5.69± 0.042	5.38± 0.020
HAOC	400	4.37± 0.038	4.94± 0.028	5.49± 0.025	5.95± 0.052	6.38± 0.024	6.06± 0.020	5.78± 0.027

Statistical analysis: Data are expressed as mean ± SEM (standard error of mean) for five animals. The difference among means has been analyzed by student- t test.

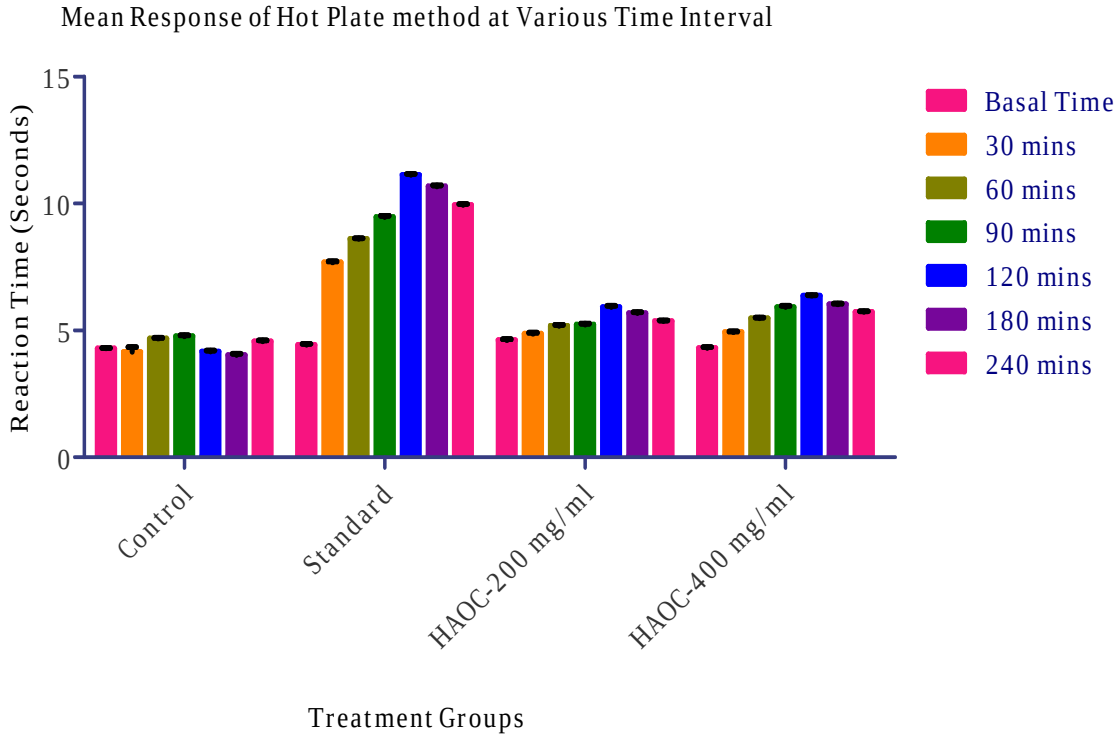

FIGURE 3:- Mean Response of Hot Plate method at Various Time Interval

Tail flick method:

TABLE 5:- Mean TFL of Tail flick method at Various Time Interval

Treatment	Dose (mg/kg)	Mean Basal Time (Seconds)	Mean Response of Tail Flick method at Various Time Interval (in minutes)					
			30	60	90	120	180	240
Distilled water	0.5ml	4.19± 0.029	3.76± 0.041	4.68± 0.046	4.77± 0.041	4.57± 0.035	4.23± 0.047	4.07± 0.037
Aspirin	10	4.29± 0.045	7.88± 0.042	8.41± 0.042	9.47± 0.046	11.13± 0.036	10.68± 0.037	9.94± 0.041
HAOC	200	4.52± 0.057	4.41± 0.010	4.52± 0.034	5.32± 0.013	5.77± 0.020	5.52± 0.022	5.38± 0.041
HAOC	400	4.21± 0.018	4.79± 0.016	4.89± 0.005	5.48± 0.025	6.82± 0.012	6.16± 0.010	6.07± 0.035

TFL=Tail Flick Latency.

Statistical analysis: Data are expressed as mean ± SEM (standard error of mean) for five animals. The difference among means has been analyzed by student- t test.

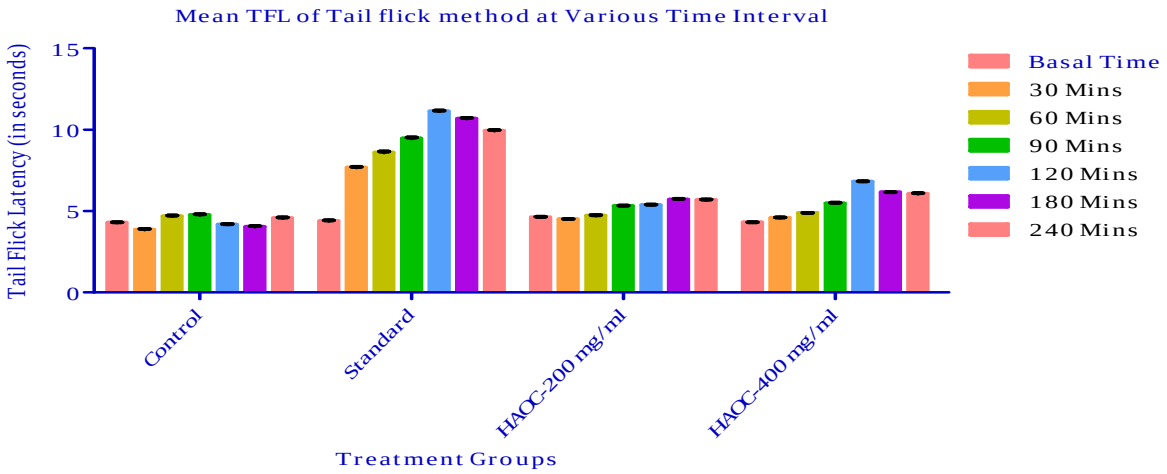

FIGURE 4:-Mean Response of Tail flick method at Various Time Intervals

Acetic acid writhing test:

TABLE-6: Mean writhes of Acetic acid writhing method

Treatment	Dose (mg/kg)	No. of Writhes	% inhibition
Distilled Water	10ml/Kg	76.66± 1.55	_
Aspirin	5	42.15± 1.07	62.73%
HAOC	200	28.67± 0.68	45.03%
HAOC	400	31.08± 0.88	59.49%

Statistical analysis: Data are expressed as mean ± SEM (standard error of mean) for five animals. The difference among means has been analyzed by student- t test.

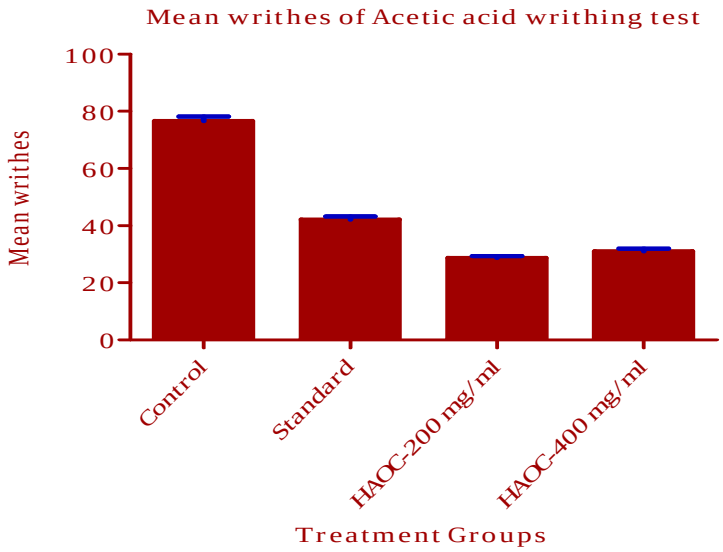

FIGURE 5:- Mean writhes of Acetic acid writhing method

DISCUSSION

Phytochemical screening

Phytochemical study shows that flavones and flavonoids are mostly present in hydro-alcoholic extract of *Ocimum canum.* Flavonoids exert their antioxidant effects (countering inflammatory, bacterial, viral, microbial, hormonal, carcinogenic, neoplastic and allergic disorders) by neutralizing all types of oxidizing radicals including the super oxide and hydroxyl radicals and by chelation. A chelator binds to metal ions in our bodies to prevent them from being available for oxidation. Also they inhibit oxidation enzymes in cells.Flavonoids also act as powerful chain breaking antioxidants due to the electron donating capacity of their phenolic groups. Flavonoids reduce the oxidation of low density lipoproteins and also prevent platelet aggregation by inhibiting the activity of the enzyme cyclooxygenase (V. Naveen Kumar H H*et al*., 2009)

In vitro study

DPPH radical scavenging assay

The degree of reduction in absorbance measurement by *Ocimum canum*is indicative of the radical scavenging (antioxidant) power of the plant. The study showed that the hydro-alcoholic extract have the proton-donating ability and can serve as free radical inhibitors or scavenger, acting possibly as primary antioxidant.

Hydrogen peroxide radical scavenging study:

The scavenging of hydrogen peroxide by the standard (ascorbic acid) and extract after incubation for 10 minutes increased with increased concentration. *Ocimum canum* hydro-alcoholic extracts exhibited higher hydrogen peroxide scavenging activity thanascorbic acids at similar concentrations. The hydro-alcoholic OC extracts have high electron-donating abilities.

In vivo study

For the purpose of investigation of analgesic activity of this plant, we used three methods, the hot plate method, tail flick method and acetic writhing method.*Ocimum canum*is rich in flavonoids, thus the results indicate that hydro-alcoholic leaf extract of *Ocimum canum*have potent antioxidant activity and also the active principles responsible for biological activity are present. The test drug *Ocimum canum* extract (hydro-alcoholic) has

been used in the dose of 200 and 400 mg/kg body weight.Analysis of tables and figures shows that the Aspirin group has significant effect as compared to distilled water treated group, whereas the hydro-alcoholic extract of *Ocimum canum*also have significant effects when compared with distilled water treated group and it can be chosen as primary analgesic.

CONCLUSION

The present study shows that hydro-alcoholic extract of *Ocimum canum* in the doses of 200 and 400mg/kg are able to produce a consistent reduction in algesia/ nociception.Further the extracts have also shown presence of active constituents responsible for various biological activities. Though they didn't produce effect as their respective standard but they still can be chosen as primary analgesic.

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