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Evaluation Of Anti-Aggressive Potential Of Aqueous Flowers Extract Of Hibiscus Rosa- Sinensis In Mice

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ABSTRACT

The present study was on the evaluation of anti-aggressive potential of aqueous flowers extract of *hibiscus rosa- sinensis* in mice. The flowers extract of the *Hibiscus rosa- sinensis* were collected from Amsar Private Limited, Himanchal Pradesh. The flowers extract was identified by the Testing Chemist (S. Paliwal). Amsar Private Limited, Himanchal Pradesh. Swiss Albino mice (20-30g) of male and female of approximate 9–12-week-old were procured from animal house of Rameshwaram Institute of Technology and Management, Lucknow, Uttar Pradesh. The experimental protocol was approved by Institutional animal ethics committee (Registration no.-1397/ac/10CPCSEA). Preliminary phytochemical screening, UV, TLC, IR and NMR spectral analysis were done. The anti-aggressive potential was evaluated using various models i.e., Resident-intruder aggression, Water competition test, Forced swimming test and Open field test. Acute and sub-acute oral toxicity studies were done. Extract was given orally at two different dose levels (200 and 400 mg/kg) once daily for three consecutive days, while CMC (Carboxy Methyl Cellulose, 1%), Diazepam (1mg/kg), was administered as positive control. In results, *Hibiscus rosa-sinensis* (200 and 400 mg/kg) on all the models produced significant anti-aggressive effects such as a reduction in vocalizations along with lowering of leaping, running, rearing, and facing each other, increased latency time to first attack, reduced spout gaining frequency along with a reduction in time spent which was found to be statistically significant compared to the control. It concluded that both the doses of aqueous flowers extract of *Hibiscus rosa- sinensis* (200 and 400mg/kg) significantly reduced the immobility time. The observed effects of diazepam in this model were qualitatively like those of *Hibiscus rosa- sinensis* which is found data statistically significant ($P<0.05$, $P<0.0001$) compared to control.

Keywords: Hibiscus rosa- sinensis, anti-aggressive, diazepam, Open field test, mice.

1. INTRODUCTION

Aggression is a deliberate series of actions that lead to harm or injury to another organism and thus constituting a major public health concern across the globe [1]. The aggressive acts may be manifested in the form of offensive and defensive behaviors. Offensive behavior is characterized by initiative of the aggressor and devastation to the opponent. Defensive behavior lacks initiative and the animal does not impose intentional damage to the opponent. In clinical settings, aggression has been classified into two more specific subtypes, such as proactive and reactive aggression [2]. Proactive aggression is overcontrolled, planned, predatory and driven by reward contingencies, whereas reactive aggression is generally characterized by an over aroused and impulsive response to a perceived threatening stimulus, with a single goal of reducing or eliminating the perceived threat. Offensive aggression in animals possesses many of the characteristic features of reactive aggression in human beings including impulsive responses and neurochemical abnormalities. The use of animal models of aggression affords the possibility of assessing the effects of drugs on specific types of aggression [3].

Plant profile: Hibiscus rosa-sinensis

The evergreen Hibiscus *rosa sinensis* can either be a shrub or a small tree up to 5 meters in height. There are five or seven lobes on the leaves, each of which is further divided into smaller lobes [4]. The flowers were used to treat epilepsy, diabetes, leprosy, and bronchial catarrh [5].

a. Leaves

b. Flowers

Fig. 1 *Hibiscus rosa-sinensis*

Taxonomy [6]
Kingdom- Plantae
Division- Megnoliophyta
Class- Magnoliopsida
Subclass- Dilleniidae
Order- Malvaceae
Genus- *Hibiscus*
Species- *Hibiscus rosa sinensis*

Flowers were studied for their potential to treat heart disease in ancient Indian medical texts. Petals were used to treat thinning hair, prevent greying, and treat and prevent problems of the scalp. As a natural emollient, it was incorporated into hair washes, treatments, and vinegar rinses [7][8]. The present study was on the evaluation of anti-aggressive potential of aqueous flowers extract of *Hibiscus rosa- sinensis* in mice.

2. MATERIALS AND METHODS

Chemicals and Instruments

Normal saline, Formalin saline, Picric acid, Ethylene diamine tetra acetic acid (EDTA), Sodium hydroxide solution, Dilute acid, Zinc dust, Concentrated hydrochloric acid, Picric acid, Ferric chloride solution, Ninhydrin solution, Alcoholic a-naphthol, Concentration Sulphuric acid, Barfoed's reagent, Dilute sulphuric acid, Fehling's solution, Dragendoff's reagent, Mayer's reagent, Wagner's reagent, Tannic acid solution, Chloroform, Dilute ammonia, Pot. hydroxide solution, N-butanol, Acetic acid, Water, Toluene, Ethyl acetate, Diazepam.

Systronics Double Beam Spectrophotometer, Perkin Elmer Spectrum Version Infrared-Spectrophotometer and NMR Instrument.

Procurement of extract

The flowers extract of the *Hibiscus rosa- sinensis* were collected from Amsar Private Limited, Himanchal Pradesh. The flowers extract was identified by the Testing Chemist (S. Paliwal). Amsar Private Limited, Himanchal Pradesh.

Procurement of Experimental Animals

Swiss Albino mice (20-30g) of male and female of approximate 9–12-week-old were procured from animal house of Rameshwaram Institute of Technology and Management, Lucknow, Uttar Pradesh. The animals were maintained in clean polypropylene cages with 12 h light and dark cycle at a temperature of 25-30^{O}C and a humidity of 50 to 60%. The animals were acclimatized to laboratory condition for one week before starting the experiment. The animals were fasted for at least 12 h before on set of each activity. The experimental protocol was approved by Institutional animal ethics committee (Registration no.-1397/ac/10CPCSEA).

Preliminary Phytochemical Screening [9][10]

Detection of Flavonoid

Alkaline Reagent Test: To the test solution add few drops of sodium hydroxide solution, intense yellow colour is formed which turns to colourless on addition of few drops of dilute acid indicate presence of flavonoid.

Zinc Hydrochloride Test: To the solution add a mixture of zinc dust and concentration hydrochloric acid. It gives red colour after few minutes.

Detection of Cardiac Glycoside

Baljet's Test: Treat the test solution with picric acid or sodium picrate, orange colour is formed.

Detection of Saponins

Froth Formation: Place 2 ml solution of drug in water in a test tube, shake well, stable froth (foam) is formed.

Detection of Tannins

Ferric Chloride Test: Treat the extract with ferric chloride solution, blue colour appears if hydrosylable tannins are present and green colour appears if condensed tannins are present.

Detection of Amino Acid

Ninhydrin Test: To the test solution add Ninhydrin solution, boil, violet colour indicate presence of amino acid

Detection of Carbohydrate

Molich's Test: To the test solution add few drops of alcoholic a-naphthol, then add few drops of concentration sulphuric acid through sides of test tube, purple to violet colour ring appears at the junction.

Barfoed's Test: 1 ml of test solution is heated with 1 ml of Barfoed's reagent on water bath, if red cupric oxide formed, monosaccharide is present. Disaccharides on prolong heating (about 10 min.) may also cause reduction, owing to partial hydrolysis to monosaccharides.

Detection of Steroid and Triterpenoids

Salkowski Test: Treat the extract with few drops of concentrated sulphuric acid, red colour at lower layer indicate presence of steroids and formation of yellow coloured lower layer indicate presence of triterpenoids.

Detection of Glycoside

Test A: Extract 200 mg of drug with 5 ml of dilute sulphuric acid by warming on a water bath. Filter it. Then neutralize the acid extract with 5% solution of sodium hydroxide. Add 0.1 ml of Fehling's solution A and B until it becomes alkaline (test with pH paper) and heat on a water bath for 2 minutes. Note the quality of red precipitate formed and compare with that of formed in Test B.

Test B

Extract 200 mg of the drug using 5 ml of water instead of sulphuric acid. After boiling add equal amount of water as used for sodium hydroxide in the above test. Add 0.1 ml Fehling's solution A and B until alkaline (test with pH paper) and heat on water bath for 2 minutes. Note the quantity of red precipitate formed. Compare the quantity of precipitate formed in Test B with that of formed in Test A. If the precipitate in Test B greater than in Test Glycoside may be present. Since Test B represent the amount of free reducing sugar already present in the crude drug, whereas Test A represent free reducing sugar plus those related-on acid hydrolysis of any glycoside in the crude drug.

Detection of Alkaloids

Tannic Acid Test: Alkaloids give buff colour precipitate with tannic acid solution.

Mayer's Reagent Test: Alkaloids give cream colour precipitate with Mayer's reagent.

Wagner's Reagent Test: Alkaloids give yellow reddish=brown precipitate with Wagner's reagent.

Dragendoff's Reagent Test**:** Alkaloids give reddish brown precipitate with Dragendoff's reagents.

Hager's Reagent Test: Alkaloids give yellow precipitate with Hager's reagent (Saturated solution of picric acid).

Detection of Coumarin Glycoside

Place a small amount of sample in test tube and cover test tube with a filter paper moistent-c with dilute sodium hydroxide solution. Place the covered test tube on water bath for several minutes. Remove the paper and expose to ultraviolet (UV) light, the paper shows green fluorescence.

Detection of Anthroquinone Glycoside

Borntrager's Test: Boil the test material with 1 ml of sulphuric acid in a test for five minutes. Filter while hot. Cool the filtrate and shake with equal volume of dichloromethane or chloroform. Separate the of dichloromethane or chloroform and shake it with half of its volume of dilute ammonia. A rose pink to red colour is produced in the ammoniacal layer.

Hydroxy-Anthroquinone Test: Treat the sample with potassium hydroxide solution red colour is produced.

Detection of Naphthoquinone

Dam-Karrer Test: To the chloroformic plant extract add 10% pot hydroxide solution blue colour develops.

Thin Layer Chromatography (TLC) of Extract

- **Stationary Phase**

The stationary phase used was TLC silica gel 60_{F254} aluminium sheets (Merk, Germany).

- **Preparation of Sample Extract**

The sample plant extract was prepared simply by just dissolving the required quantity of the extract in aqueous.

- **Preparation of Solvent System**

Solvent system incorporated for all this plant parts extracts are common. Water: Acetic acid: N-butanol (5:4: and Toluene: Ethyl acetate: Acetic acid (9.5:8:5.2) in this ratio are used solvent system.

- **Preparation of Spraying Reagent**

Iodine chamber is used to detect the presence of flavonoid compounds. It was simply prepared by iodine keep in the iodine chamber.

Ultra-Violet (UV) Analysis of Extract

Hibiscus rosa sinensis flower extract was dissolved in ethanol in microgram amounts. Shimadzu 1601 UV-spectrophotometer was used to scan their UV-visible spectra between 200 and 800 nm. Each time, the base line was set against the solvent used to make the specific extract solution. Peaks of maximum absorption were noticed in the scanned spectra, which were recorded [11].

Infrared (IR) Analysis of Extract

Hibiscus rosa sinensis (10 mg) flower extract was combined with 100 mg of dry potassium bromide (KBr) and compressed to create a salt disc. The disc was then spectrophotometrically read using a Shimadzu FTIR-8101 spectrophotometer in the 400–4000 cm-1 range (Vmax in cm-1). Each extract's frequencies of the various constituents were examined [12].

Nuclear Magnetic Resonance (NMR) Analysis of Extract

NMR is the branch of spectroscopy dealing with the absorption of radio-frequency radiation by substances held in a magnetic field. Absorption results from interaction of radiation with magnetic moment of nuclei in the sample and it occurs at different frequencies for nuclei with chemically different environments within a molecule. NMR has been an extremely important tool for elucidation of molecular structure, especially the stereochemistry and configuration. The technique reveals position of protons in a complex molecule. NMR has found many applications in the determination of impurities and minor components in mixtures because of ease, speed and specify of the analysis (Kokate, S.K.,2004). NMR spectra of aqueous extract of plant parts were done using instrument (CDRI, Lucknow).

Acute Oral Toxicity Study

The acute toxicity study was carried out by guidelines set by OECD 423 guidelines albino female mice (25-35g) maintained under standard laboratory condition was used. A total number animals (n=3) were used which received a single dose (2000 mg/kg body weight (p.o) of herbal drug (OECD, Guidelines, 2000). Animals were kept overnight fasting prior to drug administration. After the administration of poly herbal drug, the food was with-held for 3-4 hours. Animals were observed individually at least once during the first 30 minutes after dosing, periodically during the first 24h (with special attention during the first 4 hours) and daily thereafter for a period of 14 days. Daily cage side observation included changes in skin and fur, eyes and mucous membrane (nasal) and also respiratory rate, circulatory, autonomic changes was observed. . Three different doses of 1600, 2900 and 5000 mg/kg body weight of the extract were administered to each rat. The animals were monitored for 24 hours for mortality. The LD_{50} was calculated as the geometric mean of the maximum dose that caused 0% death and the minimum dose that caused 100% death [13].

- **Hematological Analysis**

On the necropsy day, blood was withdrawn through cutting of the tail end of mice. The blood was placed into EDTA bottles for heamatological assay and in plain bottle for biochemistry determination. Hb levels in blood samples were determined by the spectrophotometric method. RBC count was determined by the visual counting method. Packed cell volume (PVC), WBC, differential and platelet counts utilizing mechanical expansion and optical magnification, augumented by supravival cell staining, the QBC II system driver's platelet count, WBC subgroups from linear measurement of the packed cell layers in a buffy coat. As in conventional microcentrifugation procedures, the PVC is also measured (QBC II Manual). The blood was filled with anticoagulated blood using a pipette. The tip of the tube was cleaned off the blood with tissue paper. The tube was sealed and gently rolled between fingers for about 5 Second. The unsealed end of the tube was slid over the tip of the prepositioned float and pushed until the float was inside the tube as for as possible. The blood tube was then centrifuged on the rotor of the QBC II centrifuge for 5 minute and read on the QBC II raeder. MCV, MCH and MCHC were calculated (Dancier & Lewis, 1991) MS-9 Heamatology Analyser.

Oral Sub- Acute Toxicity Study

In this assay albino mice (25-30 g) obtained from the institute's animal house were used. The animals housed in cages at 22ºC were starved overnight with free access to water. Three animals of female albino mice were formed. A dose limit at 2000mg/kg of *Hibiscus rosa-sinensis* dissolved in vehicle was administered usually to animals from the test group.

Following administration, the animals were closely observed during the 3h, and occasionally, thereafter, for 14 days, for toxic signs and symptoms, and death. The weight of the animals was measured daily. During the 14 days period dead animals were autopsied and at the end of the period, survivors were sacrificed to examine vital organ gross changes.

- **Histopathological Analysis**

There animals were selected randomly from group anesthetized with chloroform vapor and dissected through a central abdominal incision. The kidney, heart, liver sample were collected and immediately fixed in 10% saline- formalin in labeled sample plastic bottles. The tissue was dehydrated in graded concentration of xylene embedded in moisten paraffin wax and sectioned at 5µ. Tissue section was fixed on grease- free glass slides and stained with hematoxylene and eosine for light microscopy at 20X and 40X. Photomicrograph of some of the tissues were taken using a microscope fitted with a camera unit, and processed routinely in a colour photo laboratory (Kieman, et al, 1981).

Group design

Group I (Control): Treated with 1% CMC (Carboxy Methyl Cellulose) solution, orally.
Group II (Standard): Treated with diazepam 1 mg /kg, orally.
Group III (Test drug): Treated with aqueous extract of flowers of *Hibiscus rosa-sinensis* 200 mg/kg, orally.
Group IV (Test drug): Treated with aqueous extract of flowers of *Hibiscus rosa-sinensis* 400 mg/kg, orally.

Evaluation of anti-aggressive potential

- **Resident-Intruder Aggression**

Male Swiss albino mice (25 to 30 gm) were tested in their home cages for aggression against a smaller (25 to 30 gm) male intruder. Before the start of the experiments, each resident male mice were kept in a pair with one female mice in a polypropylene cage for 15 days, and they were randomly divided into four groups (n = 6). Drug treatment was started from the16th day onward, and only male mice of each pair were administered with CMC (1%), *Hibiscus rosa –sinensis* (200 and 400 mg/kg, orally) or diazepam (1 mg/kg B.W., orally) for 3 consecutive days. The resident female was removed from the cage 30 min prior to the start of the test. One hour after the last oral treatment, a male intruder (25 to 30 gm) was placed in the territorial cage of the resident male, and behavior of the resident male was observed for the next 15 min. During this period, the time until the first attack (in seconds), number of attacks, and duration of each attack (in seconds) were recorded by a blind observer [14].

- **Water Competition Test**

Male Swiss albino mice of (25-30gm) were paired and housed in different cages. After 6 days of acclimatization, animals were deprived of water for 23 hrs. At the end of this, pairs of water deprived animals were administered with the CMC and 60 minutes later, a water bottle having a spout was introduced such that only one animal of a pair can drink at a time. Frequency and time in seconds of spout possession were recorded for 5 minutes and the aggressive animal of the pairs was marked for identification. Animals were then provided with water for 55 minutes after which the water bottle was withdrawn for 23 hours. The test was repeated on the second day. On day-2 instead of the control drug, the aggressive animals received Diazepam (1 mg/kg B. W., orally), 200mg/kg and 400mg/kg *Hibiscus rosa-sinensis* which was administered 60 minutes prior to the recording and aggressive bursts were observed for 5 minutes [14].

- **Open Field Test**

The open field test apparatus consisted of a wooden box (68 x 68 x 45 cm^3), with a dark gray floor, subdivided into 16 equal fields. The experimental room was a sound attenuated, dark room. The open field test, illuminated with a 40W bulb, focusing on the field from a height of about 100 cm, was placed in the experimental room. After 45 min of treatment, the mice were placed individually in a corner square of the open field test and the ambulation (the number of squares crossed at periphery), total locomotion (total number of squares travelled) and activity in the centre (number of central squares crossed) was recorded for 5 min [15].

- **Forced Swimming Test**

Mice of either sex were individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water at 25±1°C. The immobility time, defined as the absence of escape-oriented behaviors, such as swimming, was scored during 6min with the help of stop-watch. All the mice of either sex were divided in four different groups. The first group assigned as control receiving only vehicle (1% CMC). The other groups received acute dose of extracts. The total duration of immobility was recorded during the last 6 min of the 10 min period. Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. A decrease in the duration of immobility is indicative of an anti-aggressive like effect. For the next exposure extracts of *Hibiscus rosa-sinensis* flowers, FST of repetitive doses of crude extracts were

assessed after 3 days of treatment within 30 min after the last dose of administration. During the test session, the immobility time was recorded. The mice were considered immobile when neither hind leg was moving; the mice were slightly hunched forward [16].

3. RESULTS AND DISCUSSION

Preliminary Phytochemical Screening

- **Qualitative Chemical Tests**

The preliminary tests of aqueous extract of red flowers extract of *Hibiscus rosa- sinensis* shows the presence of flavonoids, saponins, glycoside, carbohydrate, coumarine glycoside. And flavonoids are major group of compounds which have the following effects such choleretic and diuretic functions, decreasing blood pressure, reducing the viscosity of the blood and stimulating intestinal peristalsis.

Table 1. Phytochemical Screening of aqueous flowers extract of *Hibiscus rosa-sinensis*

Chemical Constituent	Test	Result
Flavonoid	Alkaline reagent test	Present
	Zinc hydrochloric test	Absent
Cardiac Glycoside	Baljet's test	Absent
Saponins	Froth formation	Present
Tannin	Ferric chloride	Absent
Amino acid	Ninhydrin solution	Absent
Carbohydrate	Molisch's Test	Present
	Barfoed Test	Absent
Steroid & Triterpenoids	Salwoski	Absent
Glycoside	Test A & B	Present
Alkaloids	Tannic acid	Absent
	Mayer's test	Absent
	Wagner's test	Absent
	Drangdroff's reagent test	Absent
	Wagner's test	Absent
Coumarin glycoside	Ferric chloride test	Present
Anthroquinone glycoside	Borntrager's test	Absent
	Hydroxy-anthroquinone	Absent
Napthoquinone	Dam-Karrer test	Absent

Ultra-Violet Spectroscopy

UV-Visible spectrum of flowers extract of *Hibiscus rosa- sinensis* in (water: acetic acid: N- butanol) and (aqueous) shows the presence of quercetin and isoflavones in maximum wavelength at (278nm) and (234nm) at absorbance 0.288 and 1.544 (Oyvind, M., et al).

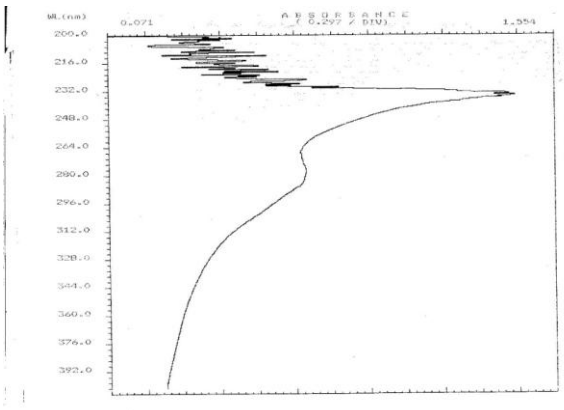


Fig 2. UV Spectrum of aqueous flowers extract of *Hibiscus rosa-sinensis*

Table 2. Ultra-Violet Spectroscopy of flowers extract of *Hibiscus rosa- sinensis* in different solvent system

S. No.	Solvents System	Absorbance	Wavelength (nm)	Chemical Constituent
1.	Water: Acetic acid: N- Butanol	0.288	278.0	Quercetin
2.	Aqueous	1.544	234.4	Isoflavones

Infrared Spectroscopy

Infra -red spectrum of flowers extract of *Hibiscus rosa- sinensis* in aqueous shows presence of alcoholic, phenolic and aromatic (benzene) functional group i.e. O-H, C-H, C=C stretching at the band frequency 3400-3100, 2900-2840, 1660 cm^{-1}.

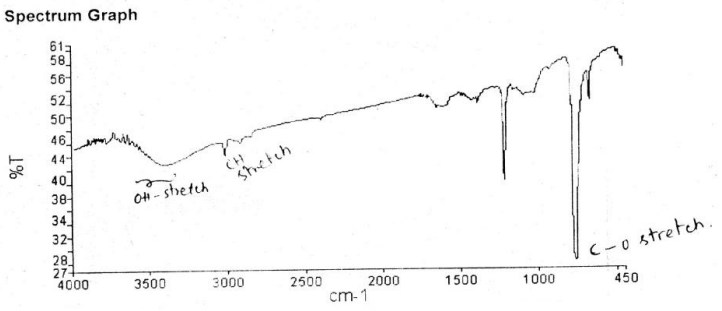


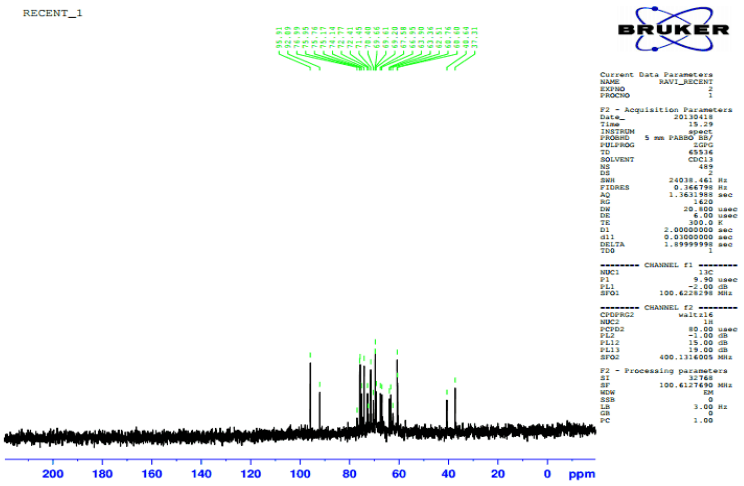
Fig 3. IR Spectrum of aqueous extract of *Hibiscus rosa-sinensis*

Table 3. Infrared spectroscopy of flowers extract of *Hibiscus rosa-sinensis*

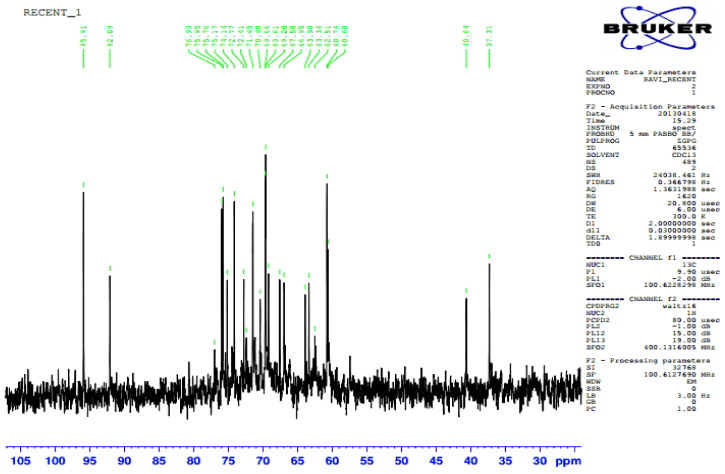
Band frequency cm^{-1}	Band Shape	Bond	Functional group
3400 - 3100	Broad	O-H Stretch	Alcoholic, Phenolic
2900 -2840	Sharp	C-H Stretch	Aliphatic
1660	Weak	C=C Stretch	Aromatic (benzene)

Nuclear Magnetic Resonance Spectroscopy

NMR spectrum of flowers extract of *Hibiscus rosa- sinensis* in aqueous shows in (Fig 4. a & b).



(a)



(b)

Fig 4. (a) and (b): NMR Spectra of aqueous flowers extract of *Hibiscus rosa- sinensis*

Thin Layer Chromatography

Thin layer chromatography of flowers extract of *Hibiscus rosa-sinensis* in different solvent system water: acetic acid: N-butanol and toluene: ethyl acetate: acetic acid in the ratio (5: 1: 4) and (9.5: 8: 5.2) shows that presence of flavonoids i.e. quercetin is retention factor (Rf) value 0.76 and 0.50.

Table 4. Thin Layer Chromatography of Flowers extract of *Hibiscus Rosa-sinensis* in different solvent system

Solvent System	Ratio	Rf Value	Result
Water: Acetic acid: N-Butanol	5: 1: 4	0.76	Flavonoid (Quercetin)
Toluene: Ethyl acetate: Acetic acid	4.7: 4 : 2.6 or 9.5: 8: 5.2	0.50	Flavonoid (Quercetin)

(a) **(b)**

(Water: Acetic acid: N-butanol) **(Toluene: Ethyl acetate: Acetic acid)**

Fig 5. TLC plates of flowers extract of *Hibiscus rosa-sinensis* in different solvent system

Acute Toxicity Study

The aqueous extract of *Hibiscus rosa-sinensis* were screened for acute toxicity study by OECD guideline for the determination of LD50 values. The result shows that extract LD50 was found to be to 200 and 400 mg /kg. Hence aqueous extract of flowers of *Hibiscus rosa-sinensis* dose 200 and 400 mg/kg, selected for anti-aggressive activity.

Animal Body Weight

Table 5. Observation for animal's body weight during toxicity study

Groups	**Mice**	**Animal body weight**		
Control	**Days**	**Initial (1st day)**	**Middle (3rd day)**	**Last (6th day)**
	Head	24 gm	22 gm	23.4 gm
	Back	22 gm	24 gm	23.5 gm
	Tail	27 gm	22 gm	20.4 gm
Treated	**Head**	25 gm	25 gm	24.9 gm
	Back	30 gm	29 gm	29.1 gm
	Tail	28 gm	27 gm	25 gm

Behaviour Parameters

Table 6. Different behaviour parameter of animals in toxicity study for 14 days

Days	**30 Min.**			**4 Hrs.**			**24 Hrs.**			**48 Hrs.**			**1 Week**			**2 Weeks**		
Mice	**H**	**B**	**T**	**H**	**B**	**T**	**H**	**B**	**T**	**H**	**B**	**T**	**H**	**B**	**T**	**H**	**B**	**T**
Appearance	Normal			Normal			Normal			Normal			Normal			Normal		
Activity	Normal			Normal			Normal			Normal			Normal			Normal		
Fur coat	Normal			Normal			Normal			Normal			Normal			Normal		
Mucus membrane	Normal			Normal			Normal			Normal			Normal			Normal		

Body orifice	Normal	Normal	Normal	Normal	Normal	Normal
Eye lacrimation	Normal	Normal	Normal	Normal	Normal	Normal
Grooming	Normal	Normal	Normal	Normal	Normal	Normal
Pupil size/Heart rate	Normal	Normal	Normal	Normal	Normal	Normal
Excreta urine faeces	Normal	Normal	Normal	Normal	Normal	Normal
Respiration	Normal	Normal	Normal	Normal	Normal	Normal
Piloexcreta	Normal	Normal	Normal	Normal	Normal	Normal
Convulsion	Normal	Normal	Normal	Normal	Normal	Normal
Sedation	Normal	Normal	Normal	Normal	Normal	Normal
Catotonia	Normal	Normal	Normal	Normal	Normal	Normal
Morbidity	Normal	Normal	Normal	Normal	Normal	Normal
Mortality	Normal	Normal	Normal	Normal	Normal	Normal

Haematological Analysis

Hematological values measured showed a significant elevation of lymphocytes level, Hb and WBC level in treatment group. The value of MCV was significant increased as compared with the control group. Other hematology values, RBCs, MCH, MCHC, Lymphocyte no and PLT were not significantly different as compared to the control mice and they remained within normal limits (control values). Hematology data are presented in (Tables 3.3.3.1) respectively.

Table 7. Effect of aqueous extracts of *Hibiscus rosa-sinensis* on some hematological parameters

Blood Parameters	**Control Group**	**Extract Treated Group (2000mg/kg)**
Heamoglobulin	14.43± 0.11***	14.4± 0.28***
Total Leukocyte Count	12700± 47.63***	40666.66± 14.692.33***
Differential Leukocyte Count		
Granulocyte	15.66± 1.34***	22.33± 2.37***
Lymphocyte	82.33±1.59***	72.66± 2.51***
Monocyte	0.02± 0.71***	1.66± 0.15***
RBC Count	4.83±0.065***	4.23± 0.062***
Platelet Count	4.3± 0.12***	4.16± 0.207***
MCV	89.13± 1.62***	99.93± 0.57***
MCH	29.9± 0.37***	32.96± 0.175***
MCHC	33.53± 0.237***	32± 0.425***
PCV	43± 0.47***	44± 0.608***

Values represent mean ±SEM, n=3, (Student's t-test).

***= Statistical significance of P< 0.05, P< 0.0001 compared to control.

Histopathological Parameter

The photomicrographs of liver, kidney, heart and brain, lungs sections from control and experimental mice stained with hematoxylin and eosin are shown below. The tissue sections of the experimental animals were essentially normal when compared with the control sections.

Table 8. Observation for Organ weight of animals

Parameter	Body weight (gm)	Liver	Kidney		Lungs	Brain	Spleen	Heart	Ovary
			R	L					
Absolute organ weight	28 gm	1.380	0.210	0.205	0.195	0.340	0.185	0.125	0.115
Relative organ weight	28 gm	4.928	0.750	0.732	0.696	1.214	0.660	0.446	0.410

Effect of aqueous flowers extract of *Hibiscus rosa sinensis* in acute toxicity study

In acute toxicity study, aqueous flowers extract of *Hibiscus rosa sinensis* was tested and analyzed through histopathological data. In this order, lungs, liver, spleen, kidney, heart and brain were utilized.

Control **Treated**

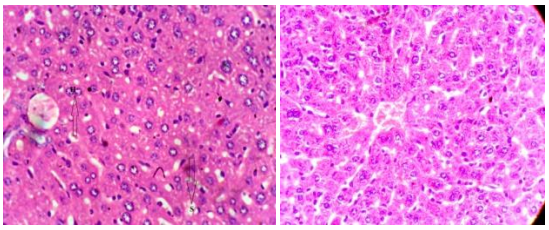


Fig 6. Liver shows normal histopathological report compare to control

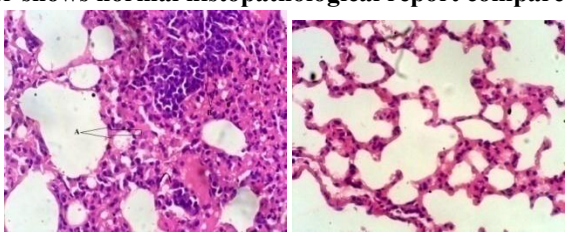


Fig 7. Lungs shows normal histopathological report compare to control

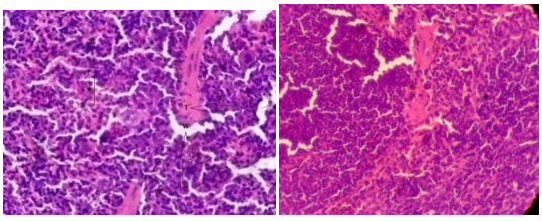


Fig 8. Spleen shows normal histopathological report compare to control

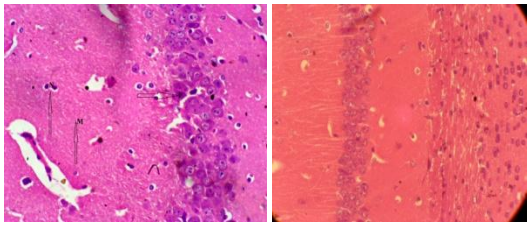


Fig 9. Brain shows normal histopathological report compare to control

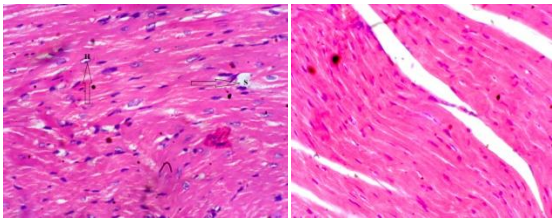


Fig 10. Heart shows normal histopathological report compare to control

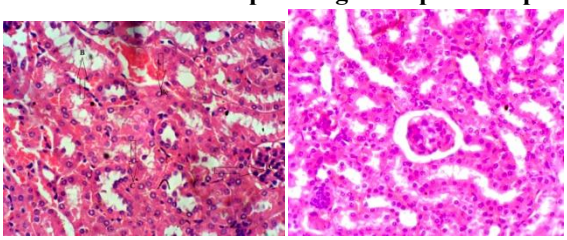


Fig 11. Kidney shows normal histopathological report compare to control

Evaluation of Pharmacological potential

- **Resident-Intruder Aggression**

Both the doses of aqueous extract of *Hibiscus rosa-sinensis* (200, and 400 mg/kg) significantly reduced the latency period of first attack and significantly reduced frequency of fighting bouts and frequency of lateral threats. The total duration of fighting was also reduced significantly by both the doses (200mg/kg and 400 mg/kg) of *Hibiscus rosa-sinensis*. The observed effects of diazepam in this model were qualitatively similar to those of *Hibiscus rosa-sinensis* which is found data statistically significant (P<0.05, P<0.0001) compared to control and latency of first attack is no significant data (P> 0.05)

Table 9. Effects of aqueous extract of *Hibiscus rosa-sinensis* and Diazepam on aggression related behaviour in the resident- intruder aggression test

Groups	Treatment (Dose)	Latency of first attack	Total Duration of fighting (Sec)	No. of fighting bouts	No. of Lateral threats
Control	**CMC (1%)**	3.80 ± 0.80	72.60 ± 13.06	27.99 ± 5.19	7.80 ± 1.47
Standard	**Diazepam (1 mg/Kg)**	3.00 ± 0.60	22.60 ± 4.29***	17.80 ± 3.35***	2.20 ± 0.42***
Test-1	**Hibiscus rosa-sinensis (200 mg/kg)**	1.80 ± 0.35	9.00 ± 1.95***	6.80 ± 1.37***	1.40 ± 0.26***
Test-2	**Hibiscus rosa-sinensis (400 mg/kg)**	1.80 ± 0.36	5.88 ± 1.17***	4.00 ± 0.88***	1.60 ± 0.32***

Values represent mean ±SEM, n=5, (One way ANOVA followed by Dunnett's t- test).

***= Statistical significance of P< 0.05, P< 0.0001 compared to control.

- **Water Competition Test**

Aqueous extract of *Hibiscus rosa- sinensis* at a dose of 200mg/kg and 400mg/kg produced a significant reduction in duration of spout possession and frequency of spout possession which was found to be statistically significant (p<0.05, P<0.0001) compared to the control.

Table 10. Effects of aqueous extract of *Hibiscus rosa-sinensis* and Diazepam on aggression related behaviour in the water competition test

Groups	Treatment (Dose)	Duration of spout possession	Frequency of spout possession
Control	**CMC (1%)**	242.2 ± 44.56	16.4 ± 3.03
Standard	**Diazepam (1 mg/kg)**	116.6 ± 25.24***	7.20 ± 1.30***
Test-1	**Hibiscus rosa-sinensis (200 mg/kg)**	79.40 ± 14.65***	8.40 ± 1.54***
Test-2	**Hibiscus rosa-sinensis (400 mg/kg)**	59.20 ± 10.67***	5.80 ± 1.21***

Values represent mean ±SEM, n=5, (One way ANOVA followed by Dunnett's t-test).

***= Statistical significance of P< 0.05, P< 0.0001 compared to control.

- **Open Field Test**

Aqueous extract of *Hibiscus rosa-sinensis* at a dose of 200mg/kg and 400mg/kg produced a significant prolonged number of squares visited in center, number of squares visited in periphery and time spent in center which was found to be statistically significant (p<0.05, P<0.01, P<0.0001) compared to the control and rearing time is no significant data (P> 0.05).

Table 11. Effects of aqueous extract of *Hibiscus rosa-sinensis* and Diazepam on aggression related behaviour in the open field test

Groups	Treatment (Dose)	No. of square visited in center	No. of square visited in periphery (Sec)	Time spent in center (Sec)	Rearing Time (Sec)
Control	CMC (1%)	0.40 ± 0.17	298 ± 53.45	1.20 ± 0.52	0 ± 0
Standard	Diazepam (1 mg/kg)	1.80 ± 0.35***	293.20 ± 52.45***	6.80 ± 1.57***	2.20 ± 0.45**
Test-1	Hibiscus rosa-sinensis (200 mg/kg)	4.20 ± 0.80***	283.40 ± 50.74***	16.60 ± 3.77***	5.60 ± 1.18**
Test-2	Hibiscus rosa-sinensis (400 mg/kg)	9.40 ± 1.70***	267.40 ± 47.87***	32.60 ± 6.12***	12.00 ± 2.54**

Values represent mean ±SEM, n=5, (One way ANOVA followed by Dunnett's t-test).

,*= Statistical significance of P< 0.05, P<0.01, P< 0.0001 compared to control.

- **Forced Swimming Test**

Both the doses of aqueous extract of *Hibiscus rosa-sinensis* (200, and 400mg/kg) significantly reduced the immobility time.

The observed effects of diazepam in this model were qualitatively similar to those of *Hibiscus rosa- sinensis* which is found data statistically significant (P<0.05, P<0.0001) compared to control.

Table 12. Effects of aqueous extract of *Hibiscus rosa-sinensis* and Diazepam on aggression related behaviour in the forced swimming test

Groups	Treatment (Dose)	Immobility Time
Control	**CMC (1%)**	216.20 ± 38.76
Standard	**Diazepam (1 mg/kg)**	140.0 ± 28.04***
Test-1	**Hibiscus rosa-sinensis (200 mg/kg)**	69.20 ± 13.72***
Test-2	**Hibiscus rosa-sinensis (400 mg/kg)**	41.40 ± 8.42***

Values represent mean ±SEM, n=5, (One way ANOVA followed by Dunnett's t-test).

***= Statistical significance of P< 0.05, P<0.0001 compared to control

The preliminary tests of aqueous extract of red flowers extract of *Hibiscus rosa-sinensis* shows the presence of flavonoids, saponins, glycoside, carbohydrate, coumarin glycoside. And flavonoids are major group of compounds which have the following effects such choleretic and diuretic functions, decreasing blood pressure, reducing the viscosity of the blood and stimulating intestinal peristalsis. UV-Visible spectrum of flowers extract of *Hibiscus rosa-sinensis* in (water:acetic acid: N-butanol) and (aqueous) the presence of quercetin and isoflavones in maximum wavelength at (278 nm) and (234 nm) at absorbance 0.288 and 1.544.

Thin layer chromatography of flowers extract of *Hibiscus rosa-sinensis* in different solvent system water: acetic acid: N-butanol and toluene: ethyl acetate: acetic acid in the ratio (5: 1: 4) and (9.5: 8: 5.2) shows the presence of flavonoids i.e. quercetin is retention factor (Rf) value 0.76 and 0.50. Hematological values measured showed a significant elevation lymphocytes level, Hb and WBC level in treatment group. The value of MCV was significant increased as compared with the control group. Other hematology values, RBCs, MCH, MCHC, lymphocyte no and PLT were not significantly different as compared to the control mice and they remained within normal limits (control values). The photomicrographs of liver, kidney, heart and brain, lungs sections from control and experimental mice stained with hematoxylin and eosin are shown below.

4. CONCLUSION

The tissue sections of the experimental animals were essentially normal when compared with the control sections. Both the doses of aqueous extract of *Hibiscus rosa-sinensis* (200, and 400mg/kg) significantly reduced the latency period of first attack and significantly reduced frequency of fighting bouts and frequency of lateral threats.

It concluded that both the doses of aqueous flowers extract of *Hibiscus rosa- sinensis* (200 and 400mg/kg) significantly reduced the immobility time. The observed effects of diazepam in this model were qualitatively similar to those of *Hibiscus rosa- sinensis* which is found data statistically significant (P<0.05, P<0.0001) compared to control.

5. CONFLICT OF INTEREST

None

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