ejpmr, 2018,5(3), 331-336

SJIF Impact Factor 4.897

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

IMMUNOMODULATORY ACTIVITY OF ANTHOCYANINS FRACTION OF *CANNA INDICA*

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Article Received on 31/12/2017 **Article Revised on 19/01/2018** **Article Accepted on 09/02/2018**

ABSTRACT

Herbal drugs play an important role in curing diseases throughout the history of mankind. The study of medicinal plants has attracted many researchers, owing to the useful applications of plants for the treatment of various diseases in humans and animals. Anthocyanins are involved in a wide range of biological activities that may affect positively the health. The aim of this study is to evaluate and compare the Immunomodulatory activity of anthocyanins from *Canna indica* flowers. *Canna indica* flowers were collected in Toopranpet village of Nalgonda district. Methanolic extraction method was used for extracting anthocyanins from plant materials. From the investigation as the extract gave positive results, it was confirmed that *Canna indica* flower extracts (CIFE) consists of anthocyanins. Acute toxicity study was carried out in male Swiss albino mice. CIFE are considered to be safe up to the dose levels of 1000 mg/kg (b.w). The results of the present study suggest that CIFE (500 mg/kg *p.o*) increases the cellular and humoral immunity as indicated by an increase in clearance of carbon from blood stream in carbon clearance test.

KEYWORDS: *Canna indica*, Anthocyanins, Immunomodulatory activity and Herbal drugs.

INTRODUCTION

Herbal drugs play an important role in curing diseases throughout the history of mankind. Now medicinal plants are recognized globally as important resources for all major system of medicine, health care, nutraceuticals, phytochemicals and cosmetics. The study of medicinal plants has attracted many researchers, owing to the useful applications of plants for the treatment of various diseases in humans and animals. To date, medicinal plants have been used in all cultures as a source of medicine for the treatment of various diseases including stomach complaints, malaria, depression, cancer, AIDS.[1]

Anthocyanins are involved in a wide range of biological activities that may affect positively the health. Many of the biological properties are closely associated with the Immunomodulatory activity of anthocyanin pigments. The aim of this study is to evaluate and compare the Immunomodulatory activity of anthocyanins from *Canna indica* flowers.[2]

MATERIALS AND METHOD

Plant Material

Canna indica L. (known as Saka siri, Indian shot, Canna, Bandera, Chancle, Coyol or Platanillo and Kardal in Marathi) is a species of Canna genus, belonging to the family Cannaceae, a native of the Carribean and tropical Americas that is also widely cultivated as a garden plant. It is a perennial growing from 0.5m to 2.5m, depending on the variety. It is hardy to zone 10 and is frost tender. In the northern latitudes it is in flower from August to October, and the seeds ripen in October. The flowers are hermaphrodite. The seeds are small, globular, black pellets, hard and heavy enough to sink in water. They resemble shotgun pellets giving rise to the plants common name of Indian shot.[3]

Plant Collection

Canna indica flowers were collected in Toopranpet village of Nalgonda district and authenticated by Prof. P Suresh, Department of Botany, Government degree college Ibrahimpatnam, Rangareddy district.

CLASSIFICATION

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Super division	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Liliopsida – Monocotyledons
Subclass	Zingiberidae
Order	Zingiberales
Family	Cannaceae – Canna family
Genus	*Canna* L. – canna
Species	*Canna indica* L. – Indian shot

Figure 1. *Canna indica* plant

Figure 2. *Canna indica* Flowers

Extraction and Isolation

Methanolic extraction is the classical method of extracting anthocyanins from plant materials. This procedure involves maceration or soaking of the plant material in methanol containing a small concentration (0.01%) of mineral acid (e.g., HCl). Methanol extraction is a rapid, easy and efficient method for anthocyanin extraction. However, a crude aqueous extract with several contaminants is obtained and methanol evaporation can result in hydrolysis of labile acyl linkages, which is aggravated by the presence of HCl.[4]

Extraction and Yield

Canna indica containing anthocyanins were extracted with maceration or soaking of the plant material in methanol containing a small concentration of mineral acid (e.g., HCl). The colour, nature and percentage yields of extracts are recorded in the Table 1.

Confirmation tests for anthocyanins

From the investigation as the extract gave positive results, it was confirmed that *Canna indica* flower extracts (CIFE) consists of anthocyanins. Confirmation tests results are recorded in Table 2.

Quantification of total anthocyanins by pH differential method.

Total quantity of anthocyanins in *Canna indica* flowers was measured by using pH differential method (cyaniding-3-glucoside equivalent).

The total quantity of anthocyanins in *Canna indica* was found to be 58.41 mg/kg, 0.3mg/kg respectively with equivalent to cyanindin-3- glucoside.

Table 1: Percentage yield and physical appearance of extracts.

S.No	Plant	Percentage Dry weight	Colour	Consistency
1	*Canna indica*	2.8%	Reddish Brown	Semi solid

THIN LAYER CHROMATOGRAPHY OF ANTHOCYANINS

Solvent system used for TLC of anthocyanins

HCl: Formic acid: Water (7:51:42)

Butanol: acetic acid: water (4:1:5).

In solvent system HCl: Formic acid: Water (7:51:42) CIFE gave single spot for each extract, and in solvent system Butanol: acetic acid: water (4:1:5) CIFE gave four spots. The R_f values of the extract in two solvent systems are given in the Table 3.

Table 2: Confirmation tests of anthocyanins.

S .No	CHEMICAL TESTS	RESULT
1	**ANTHOCYANINS**	
	Aqueous NaOH	+
	Conc. HCl	+
	Mg HCl	+
2	**CARBOHYDRATES**	
	Molisch test	+
	FOR REDUCING SUGARS	
	Fehling's test	+
	Benedict's test	+
	FOR MONOSACCHARIDES	
	Barfoed's test	+
	FOR POLYSACCHARIDES	
	Iodine test	-
3	**FLAVONOIDS**	
	Shinoda test	+
	Lead acetate test	+
4	**TEST FOR POLYPHENOLS**	
	5%$FeCl_3$	+
	Bromine water test	+

Table 3: TLC analysis of anthocyanins.

Solvent system	R_f values of extracts
HCl : Formic acid : Water (7:51:42)	0.71
Butanol: acetic acid: water (4:1:5)	0.15
	0.26
	0.65
	0.84

ACUTE TOXICITY STUDY

The study was conducted on male albino mice weighing between 20-25 gm and was divided into 2 groups containing 6 animals each. They were fasted over night.

Group-I administered CIFE.

Dose levels started from 500 mg/kg (b.w).

Acute toxicity studies were performed for anthocyanin extract at dose levels starting from 500 mg/kg body weight in mice. The number of animals were surviving was observed after 24 hours for the extract. The extract did not produce any signs of mortality and behavior changes. After one week the animals were administered 1000 mg/kg (b.w) of the extract, did not produce any signs of mortality and behavior changes. After one week the animals were administered 1500 mg/ kg (b.w). The animals produced behavior changes. Hence the drugs were considered to be safe up to the dose levels of 1000 mg/kg.

IMMUNOMODULATORY ACTIVITY

Requirements: Antigen (sheep red blood cells), Normal saline, Alsever's solution, Septilin syrup, Microtiter plate (96 well plates), Plethysmometer, 1% SRBCs suspension, Carbon ink, Neubauers chamber, Gelatin, EDTA, Sodium carbonate.

Alsever's solution: Alsever's solution is an isotonic balanced salt solution consisting of glucose 2.05%, sodium chloride 0.42%, tri–sodium citrate 0.8% and citric acid 0.55%. It is routinely used as an anticoagulant/blood preservative, and permits the storage of whole blood for approximately 2 weeks in a refrigerator at 2 – 8^o C.

Antigenic material: The sheep red blood cells (SRBCs) were used as an antigenic material. The sheep blood was obtained from slaughter house collected in Alsever's solution. During the experimentation, adequate amount of SRBCs were washed 3 times with pyrogen free normal saline (0.9% w/v NaCl). The settled SRBCs were found to be 4.8×10^6 cells/ml (by haemocytometer) and used for immunization and challenge.

Carbon ink: Camel fountain pen ink purchased from local market.

Test samples

1. CIFE 250, 500 mg/kg (b.w)
2. Septilin syrup 1 ml/100 gm

CIFE was dissolved in normal saline

TREATMENT SCHEDULE FOR ASSESSING IMMUNOMODULATORY ACTIVITY

Adult male albino rats weighing 100-200 gm each were divided into six groups consisting of six animals each.

The rats were deprived of food for 24 hours with free access to water. The treatment schedule[5] for assessing Humoral Immune Response, DTH response and WBC count are given in Table 4.

Table 4: Treatment Schedule for Assessing Humoral Immune Response, DTH response, WBC count.

S .No	Group	Treatment	Dose
1	Control	Normal saline + 50 µl of SRBCs suspension	-
2	Standard	Septilin syrup + 50 µl of SRBCs suspension	1 ml/100 gm b.w p.o/7days
3	Test 1	CIFE+ 50 µl of SRBCs suspension	250 mg/kg b.w p.o/7days
4	Test 2	CIFE + 50 µl of SRBCs suspension	500 mg/kg b.w p.o/7days

HUMORAL IMMUNE RESPONSE OR HAEMAGGLUTINATION ANTIBODY TITRE

The animals were immunized by injecting 50 µl of SRBCs suspension containing 4.8 × 10^6 cells/ml intraperitoneally on day 0. Drugs of different concentrations i.e., 250, 500 mg/kg b.w were administered to the respective groups orally for 7 days. Blood samples were collected in micro centrifuge tubes from individual animal by retro orbital puncture on day 8. The blood samples were centrifuged and serum was obtained. Antibody levels were determined by the hemagglutination titer technique. Briefly equal volumes of individual serum samples of each group were pooled. To serial two fold dilutions of pooled serum samples made in 25 µl volume of normal saline, in U-bottomed 96 well microtiter plates were added 25 µl of freshly prepared 1% suspension of SRBCs in saline. After mixing, the plates were incubated at 37ºC for 2 hrs and examined visually for agglutination. The reciprocal of the highest dilution of test serum causing visible haemagglutination were taken as the antibody titer.[6]

DTH RESPONSE

After blood collection on day 8 the thickness (ml) of the right hind foot pad was measured using plethysmometer. The rats were then challenged by injection of 25µl of 4.8 × 10^6 cells/ml SRBCs subcutaneously into right hind foot pad. Foot thickness was measured again after 24 hrs after this challenge. The difference between the pre and post challenge foot thickness was taken as a measure of DTH.[7]

WHITE BLOOD CELL COUNT

After blood collection on 8 day the collected blood was sucked up to the 0.5 mark of WBC diluting pipette. The tip was cleaned and diluting fluid is sucked up to mark 11. The fluids were thoroughly mixed by rotating the pipette between the palms of two hands. A clean cover slip was placed over counting chamber. First few drops of diluting fluid were discarded. A tiny drop was collected at the tip of pipette and was touched at junction of cover slip at the slide. Fluid was drawn in to chambers by capillary action. There should not be any air bubble. Examined under 10X. Five fields were counted. Finally, the WBCs/cmm was calculated by adding the cells in the 5 groups and multiplying by 40.[8]

CARBON CLEARANCE TEST

Different concentrations of the plant extracts i.e., 250, 500 mg//kg were administered orally for 7 days. Table 5 shows the Treatment schedule for assessing carbon clearance test.

Table 5: Treatment Schedule for Assessing carbon clearance test.

S .No	Group	Treatment	Dose
1	Control	Normal saline	-
2	Standard	Septilin syrup	1 ml/100 gm b.w p.o/7days
3	Test 1	CIFE	250 mg/kg b.w p.o/7days
4	Test 2	CIFE	500 mg/kg b.w p.o/7days

At the end of seven days the rats were injected with 0.1 ml of carbon ink (Camel fountain pen ink) suspension (1.6 % v/v in 1% Gelatin, in saline) via the tail vein. Blood samples (about 50 µl) were drawn (in 0.15 % w/v disodium EDTA solution, 50 µl) from the retro orbital vein, at intervals of 2 and 15 minutes after injection. A 25-µl sample was mixed with 0.1% sodium carbonate solution (2 ml) and the absorbance was measured at 660 nm taking 0.1% sodium carbonate solution as blank.[9] The carbon clearance was calculated using the following equation:

$$\text{Carbon clearance} = \frac{\log OD1 - \log OD2}{t_2 - t_1}$$

Where,

OD1, OD2 are the optical densities at t_1 and t_2 respectively.

t_1 ---- 0 min

t_2 ---- 15 min

RESULTS AND DISCUSSION

The CIFE was screened for immunomodulatory activity to compare their potency with control.

HUMORAL IMMUNE RESPONSE, CELLULAR IMMUNE RESPONSE and WBC COUNT

Administration of CIFE has produced significant increase in humoral immune response, cellular immune response and WBC count as compared to the normal.

Haemagglutination antibody titer was used to assess humoral immune response. The results of this experiment demonstrated that both the anthocyanin extract administration significantly rise the antibody levels in dose dependent manner. Antibody molecules, a product of B lymphocytes and plasma cells, are central to humoral immune response. HA titer value of CIFE at highest dose i.e., 500 mg/kg b.w equal to the standard value. Figure 3 illustrates the hemagglutination titer value in microtiter plate.

In the present investigation SRBCs induced Delayed Type Hypersensitivity (DTH) was used to assess the effect of the fraction on cell mediated immunity. After challenge on day 8 with SRBCs significant increase in the paw edema (DTH) was observed on day 9 (Table 6).

WBC count increased with dose dependent manner, CIFE at highest dose i.e., 500 mg/kg b.w observed highest WBC count. Effect of CIFE on WBC count recorded in Table 6.

CARBON CLEARANCE TEST

The role of phagocytosis is primarily the removal of microorganisms and foreign bodies, but also the elimination of dead and injured cells. In the present study carbon clearance were significantly increased as compared to control (Table 6). Hence, increased clearance rate of carbon particles from the circulation in animals reflects the enhancement of phagocytic function of macrophages and non-specific immunity.

The present study established the immunostimulatory activity of *Canna indica* anthocyanin extract. The immunostimulatory effect produced by the anthocyanin extract may be due to cell mediated and humoral antibody mediated activation of T and B cells, increased WBC count and increased phagocytosis. CIFE showed immunostimulatory activity.

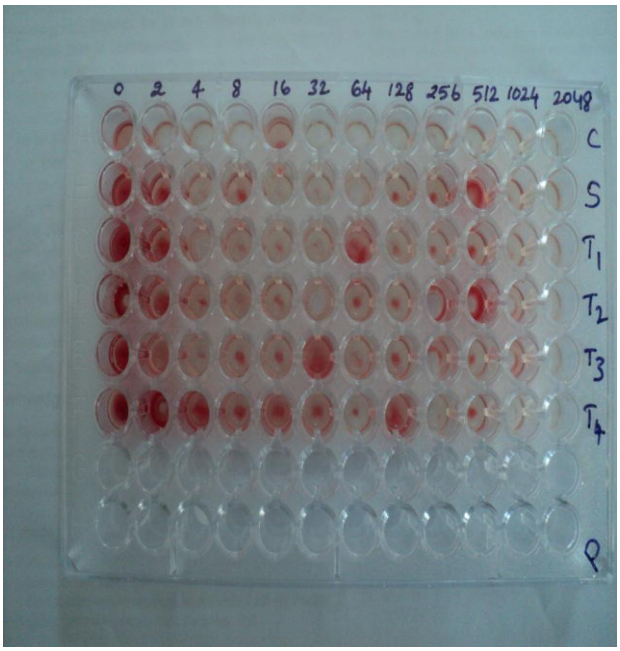

Figure 3: Microtiter plate for Humoral immune response.

Table 6: Effect of CIFE on Humoral Immune Response, DTH response, WBC count, Phagocytic response.

Group	Treatment	Dose (mg/kg)	HA titre value	DTH response (ml)	WBC count (X1000/ccm)	Phagocytic response
Normal	Normal saline	_	16	0.105±0.0083	4.13±0.145	0.010±0.0019
Standard	Septilin syrup	1ml/100gm	512	0.4±0.070***	10.83±0.78***	0.03±0.006***
Test 1	CIFE	250	64	0.145±0.064	5±0.554*	0.015±0.001*
Test 2	CIFE	500	512	0.3±0.044***	8.78±0.665***	0.021±0.002***

All values expressed as mean±S.D. n=6

*p<0.05 significant, **p<0.01 highly significant, ***p<0.001 very highly significant

SUMMARY AND CONCLUSION

Recent reports have demonstrated multiple benefits associated with the consumption of anthocyanins, which play a major role as free radical scavengers. *Canna indica* flowers are new source for anthocyanins. *Canna indica* was selected based on its anthocyanin content. The overall aim of this research work was to find effective methods for the extraction, quantification and separation of anthocyanins**,** identify the significance of the immunomodulatory activities of anthocyanins,

Anthocyanins were extracted with maceration of the plant material in methanol containing a small concentration of mineral acid (e.g., HCl). Percentage yield was found to be 2.8% respectively. Acute toxicity study was carried out in male Swiss albino mice. CIFE are considered to be safe up to the dose levels of 1000 mg/kg (b.w).

The present study evaluates the effect of administration of CIFE on the immune system in experimental animals. The carbon clearance test was carried out in rats to evaluate the effect on macrophage induced phagocytosis. The effect on humoral immunity was evaluated by studying the effect on serum immunoglobulins and by determining the haemagglutinating antibody titer in indirect haemagglutination test.

The results of the present study suggest that CIFE (500 mg/kg *p.o*) increases the cellular and humoral immunity

as indicated by an increase in clearance of carbon from blood stream in carbon clearance test. The CIFE also produced a significant increase in humoral immunity that was evident by an increase in serum immunoglobulin levels and increase in haemagglutinating antibody titer in indirect haemagglutination test, WBC count was gradually increased. CIFE at high doses stimulate the cellular and humoral mediated antibody. Based on the above observations, it can be concluded that *Canna indica* consists of high amount of anthocyanins. Therefore CIFE possess potent immunostimulant activity.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. K Ravinder Reddy, Secretary of Chaitanya Institute of Pharmaceutical Sciences, Rampur Warangal, for providing laboratory facilities and financial support.

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