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Pharmacological effects of *Raphanus caudatus* in inflammation and hematopoitic system: an *in vitro* and *in vivo* investigation

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Abstract: The pods of Radish are known as *Raphanus caudatus that* belongs to the family *Brassicaceae*. They are commonly known as Mungra or Sungra in Pakistan, while the common English name for this species is Rat-tailed radish. This variety of radish is unique and less tested for pharmacological as well as toxicological potential. In the current research, the ethanol extract of pods was assessed for anti-inflammatory potential *in vitro* and *in vivo*. Furthermore the effect of plant on hematological parameters was also investigated. For *in vitro* study, luminol-enhanced chemiluminescence method was used while *in vivo* study was carried out via Acetic acid- induced Paw Edema Test in wistar rats. The extract of *Raphanus caudatus* indicated significant anti-inflammatory effects regarding *in vitro* assay. Administered tested doses (250mg, 500mg and 1000mg/kg) of plant extract showed significant reduction in rat's paw but highest *in vivo* anti-inflammatory effect was observed at the dose of 1000mg/kg. Moreover, in the case of hematological study, noticeable elevation of white blood cell count was observed at 500 and 1000 mg/kg. However the number of platelets was reduced in dose dependent manner.

Keywords: *Raphanus caudatus*, *in vitro*, *in vivo*, anti-inflammatory, hematology.

INTRODUCTION

The significance of vegetables cannot be ignored as they have very important role in our life not only as food materials but they are used in the treatment and inhibition of different diseases. These vegetables provide major source of amino acids, vitamins, antioxidants, minerals, and fibers so they are beneficial in the development and restoration of body deficiencies (Boeing, Bechthold *et al*. 2012, Slavin and Lloyd 2012).

Raphanus sativus L. Var. *caudatus* are edible and green in color (fig. 1), processes beneficial properties for health. It grows annually and sometimes biennially, seed pods which are formed from seeds lengthen about 30-90 cm tall and can grow up to 5 feet in height and can spread up to 2 feet (Pocasap, Weerapreeyakul *et al*. 2013). Commonly in Pakistan and India it is known as Mungraa or Sungraa. The seed pod part of radish is available in Pakistan from the months of November till March and is consumed in delicious dishes (Khare 2007). Radish is a rich source of diversified types of flavonoids such as apigenin, quercetin, kaempferol, luteolin and myricetin (Lugast and Hovari 2000). Other studies also reports the presence of phenolic content in Raphanus sativus (Jamuna, Ramesh *et al*. 2015, Kim, Moon *et al*. 2015). Moreover different phenolic compounds identified in radish are reported as anti-inflammatory such as Luteolin Kaempferol, Kaempferol-7-O-rhamnoside, Kaempferol-3-glucoside-7-rhamnoside and Kaempferol-7-glucoside-3-rhamnoside (Gutiérrez and Perez 2004, Sham, Yuen *et al*. 2013). The pods of Radish are good source of Vitamin C, Vitamin B9, pyridoxine, Vitamin B2 and Vitamin E (Songsak and Lockwood 2002, Pocasap, Weerapreeyakul *et al*. 2013).

Previously various pharmacological activities of radish pods were explored by our research project (Younus and Siddiq 2017, Siddiq and Younus 2018, Younus and Siddiq 2022). In the current study, anti-inflammatory potential of this vegetable along with its effect on hematological parameters were identified by employing *in vitro* and *in vivo* experimental designs.

MATERIAL AND METHODS

Plant material

Fresh pods (10kg) of *Raphanus sativus* L.Var. *caudatus* were procured from Karachi, identified by Associate Professor of University of Karachi Department of Pharmacognosy, Dr. Mohtashim and specimen with voucher number: RSP-01-14/17 was deposited to Department of Pharmacognosy. Pods of the plant were washed using double distilled water thoroughly after which dried in shade at room temperature and saved in properly labeled air tightcontainers.

Plant material extraction

Extract of the plant material was obtained at the temperature of 60°C using Ethanol (Merck) (1:50, w/v) from soxhlet apparatus (HMFT-5/63, England)(Okoduwa, Umar *et al*. 2016) . The extraction process was performed repetitively until exhaustion (48h) and then extract obtained was filtered with autoclaved Whatman No. 1 filter paper. After filtration process of drying of filtrate was performed in a rotary evaporator (Buchi R-200,

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Switzerland) under controlled pressure and temperature of 45°C to obtain semisolid mass.

Immunomodulatory/anti-inflammatory activity – in vitro study

Chemiluminescence assay

Evaluation of immunomodulatory activity of RCE was performed with Luminol-amplified chemiluminescence method (Helfand, Werkmeister *et al*. 1982). The Test was carried out in white 96-well plates (Costar, NY, USA). A25 µl cell suspension of whole blood (diluted) in Hanks Balanced Salt Solution, containing $CaCl_2$ and $MgCl_2$ (HBSS++) (Sigma, St. Louis, USA) was incubated with 25 µl of RCE in HBSS++ at three different concentrations i.e. 1, 10 and 100µg/ml. Medium with HBSS++ and cells only were kept as control wells. Incubation was performed at 37ºC in the thermostat chamber of luminometer [Labsystems, Helsinki, Finland] for 15 minutes. Afterward, SOZ (serum opsonized zymosan) [Fluka, Buchs, Switzerland] and luminol (Research Organics, Cleveland, OH, USA), were (25µl) separately transferred to each well; however in blank wells contained with HBSS++. The ROS levels in term of RLU (relative light units) were determined by using luminometer and results were expressed as IC_{50} (50% inhibitory concentration). The tests were conducted in triplets.

In vivo study

Animals

All the animals were procured from Dow University of Health Sciences, Karachi. For anti-inflammatory activity, wistar rats weighing 200-250g and for biochemical testing on hematological parameters, albino rabbits weighing 1200-1800 g were used in the present study. All the animals were of both genders. Transparent cages were used for housing of rabbits which were housed individually in each cage while mice were housed in group of 3-6 per cage, under controlled conditions, temperature and humidity was (23±2°C), (50 to 60%) respectively in a 12/12h light and dark cycle. All Animals had access of water and fed with ad labitum. Prior to the initiation of the study all animals were observed for 1 week for acclimatization purpose to the laboratory environment and those were excluded having any infections.

Anti- inflammatory activity of Raphanus caudtus extract

Protocol of dosing

For anti-inflammatory activity of radish pods in vivo evaluation potential was done by acetic acid induced paw edema method in albino wistar rats. There were 5 groups of animals (n=10 each). All drugs were dissolved in distilled water and administered via oral route. The standard drug in the form of tablets was purchased from local market.

Acetic acid- induced paw edema test

Firstly animals of all groups were parenterally administered with 1% w/v acetic acid (0.1ml) in the right hind paw of rat's sub-plantar tissue (Bachhav, Gulecha *et al*. 2009) to induce edema. After 30 min administration of acetic acid all animals were treated orally with normal saline, standard drug (acetylsalicylic acid) and RCE (250, 500 and 1000mg/kg) respectively.

In the current study, Plethysmometer (UGO Basile, Italy) is used for the measurement of paw volume (in term of ml).The readings were recorded at "0" time (baseline), following acetic acid treatment and observed hourly till 4 hours after administration of edematogenic agent.

Edematous paw reduction was calculated by using following formula in term of percentage(Gupta, Mazumder *et al*. 2005, Rao and Najam 2016), Khobragade *et al*., 2012).

$$(\%)\ \text{Inhibition of edematous paw} = \frac{C - T}{C} \times 100$$

Where,

C = Average increase in paw volume of control rat at given time

T= Average increase in paw volume of treated rat at given time

Hematological study

Hematological parameters were observed in rabbits divided in to three groups (each contains 10 animals) i.e. RCE I, II and III (250, 500 and 1000mg/kg) were administered once daily for continuous 60 days. After dosing protocol of 60 days, 2 ml blood was collected in EDTA.K3 tubes for hematological screening. Blood samples that were collected in siliconized glass tubes (Heyns, van den Berg *et al*. 1981) investigated for hematological parameters (RBC, WBC, platelet count and hemoglobin level) by automatic Humacount plus Germany.

Ethical approval

All the research protocol, procedures, euthanasia as well as ethical clearance were approved by the BASR (Board of Advanced Studies & Research), University of Karachi (BASR number 02419/Pharm). All the selected animals were handled using ethical principles of research. Specifications described in Helsinki Resolution 1964 were adopted for handling of animals in the current research.

STATISTICAL ANALYSIS

All data analysis and statistical procedures were performed using Statistical Package for the Social Sciences (SPSS) version 20. The results were presented as mean + standard error of mean. The groups were compared by One - way ANOVA (Analysis of variance) followed by post hoc Tukey's Honest Significant Difference (HSD) test to find out statistical significance.

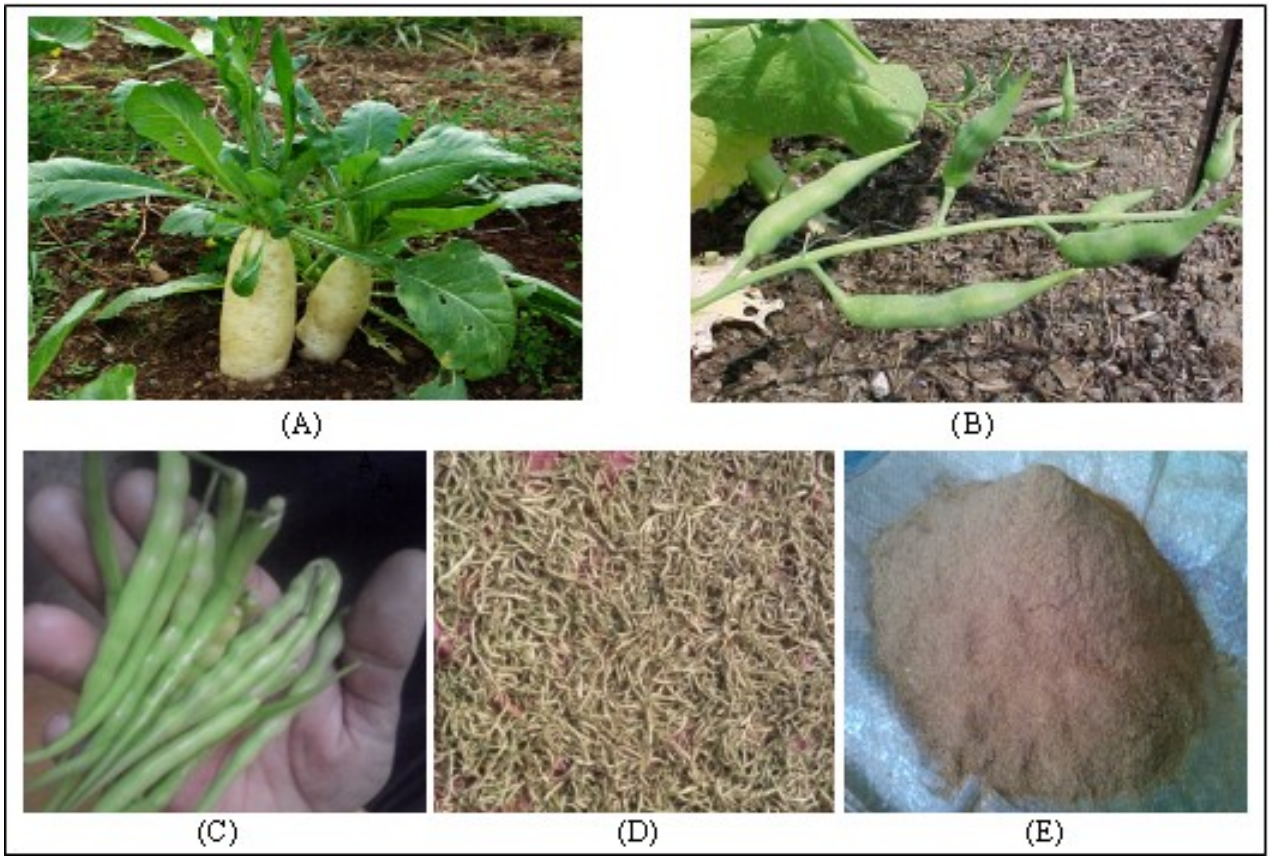

Fig. 1: Raphanus sativus L. (A) Roots (B), (C) Fresh Pods (D) Dried pods (E) Powder of dried Pods (imageswere taken from current study and flora of Pakistan).

Table 1: Dosing Protocol for *In vivo* Study

Groups	Treatment	Dose (mg/kg) p.o
Control	Normal saline	1 ml/kg
Standard	Aspirin*	300
Test group I	RCE-A	250
Test group II	RCE-B	500
Test group III	RCE-C	1000

*used as standard drug for anti-inflammatory activity.

Results obtained from current data were regarded as significant and highly significant at $p<0.05$ and $p<0.005$ respectively.

RESULTS

In vitro Anti-inflammatory / Immunomodulatory activity

The immunomodulatory activity of ethanol extract of *Raphanus caudatus* showed significant results with more IC_{50} value (37.8 ± 2.1) as compare to standard IC50 (11.3 ± 1.89) as shown in table 2.

In vivo anti- inflammatory activity

The anti-inflammatory effect of RCE was determined by acetic acid induced paw edema in rats at three different doses and acetylsalicylic acid is shown in table 3. The revealed anti-inflammatory activity of RCE showed time and dose dependent inhibition in term of (%) inhibition of paw edema and displacement in ml ± SEM.

The results showed that given extract of RCE Aat 250mg/kg revealed significant effect ($p<0.05$) on paw edema i.e. (%) inhibition of paw edema after 1h and 2h of acetic acid administration was 19.04% and 18.36%as compare to control group. The significant reduction ($p<0.005$) of rat paw edema against acetic acid administration was observed in time dependent manner at 3rd and 4thhr following administration.

Animal treated with RCE B extract presented prominent reduction in edematous paw volume induced by acetic acid in rat observed volume 1h post treatment was

3.5±0.17ml (16.66%) in comparison of saline administered group where displaced volume was i.e. 4.2±0.10ml.

Table 2: Immunomodulatory activity of *Raphanus caudatus in vitro* by Chemiluminiscence assay

Immunomodulatory study	
IC_{50} ± SEM (µg/ml)	
RCE	37.8 ± 2.1
Ibuprofen	11.3 ± 1.89

The highest tested dose in this study i.e. RCE-C administered in animals revealed prominent anti-inflammatory activity in comparison to control group animals which is approximate of standard drug acetylsalicylic acid The results indicate that animals having drug acetylsalicylic acid exhibits potent anti-inflammatory effect by decrement in rat paw edema after the acetic acid administration from 1 to 4h.

Effect of Raphanus caudatus on hematological parameters

The effect of RCE on hematological parameter (RBCs, WBCs and Platelets count) at three different doses is showed in table 4. After 60 days dosing protocol there was no significant RBCs count alterations in all three group treated with RCE (A, B and C).With respect to control in the group of RCE-A effect on WBCs count results were statistically insignificant. While group administered with RCE-B and RCE-C, WBCs count were highly significant increase ($p<0.05$, $p<0.005$) by (5.2±0.21*), (7.2±0.18**) respectively. The results of *Raphanus caudatus* on animals having RCE-A, platelet count was significant ($p<0.05$) in comparison with control group. Further, the platelet count in animals treated with RCE-B and RCE-C revealed highly significant reduction ($p<0.005$) when compared with control group. But effect on Hemoglobin levels of RCE (A, B and C) showed no significant effect in contrast to control group

DISCUSSION

Raphanus caudatus is known for its health benefits. In this study the pharmacological effects of this variety of radish have been identified in inflammation / immunomodulation. Any modification in immune system by any stimuli is called Immunomodulation. It is required in condition of immunodeficiency to combat with number of immune disorders (Gea-Banacloche 2006). In earlier study it has been showed that radish have immunomodulatory potential, which has been observed by the aqueous extract of *Raphanus niger* as it showed increase in survival rate and also provide protection against experimental models of influenza infection in mice (Prahoveanu and Eşanu 1986).

It is approved that ROS are produced after the activation PMNs and formation of ROS is known as oxidative burst.

Table 3: Effect of *Raphanus caudatus* and Aspirin on percent inhibition of paw edema in Acetic acid-induced Paw Edema Test

Treatment Groups	Displacement in 'ml'		Displacement in 'ml' and (percent inhibition) after acetic acid treatment			
	Pre-drug	Immediately after acetic acid administration 0 h	1 h	2 h	3 h	4 h
Control	2.7±0.12	2.9±0.13	4.2±0.10	4.41±0.18	4.9±0.18	4.84±0.21
Aspirin (300 mg/kg)	2.6±0.14	2.9±0.16	3.15±0.14** (25.0)	3.19±0.14** (27.66)	3.28±0.10** (33.06)	3.34±0.12** (31)
RCE-A (250 mg/kg)	3.1±0.15	2.4±0.15	3.4±0.18* (19.04)	3.6±0.16* (18.36)	3.5±0.15** (28.57)	3.62±0.11** (25.2)
RCE -B (500 mg/kg)	2.9±0.15	3.1±0.12	3.5±0.17* (16.66)	3.25±0.13** (26.30)	3.43±0.18** (30)	3.52±0.20** (27.27)
RCE-C (1000 mg/kg)	3.2±0.11	3.0±0.14	3.36±0.11** (20.0)	2.73±0.18** (38.09)	2.69±0.21** (45.10)	3.15±0.25** (35)

The values in brakettes indicate percentage edema inhibition. n =10 per group, Values are Mean ± Std. Error of Mean, RCE: *Raphanus caudatus* Extract, *p ≤ 0.05 significant versus control, **p ≤ 0.005 highly significant versus control

Table 4: Effect of *Raphanus caudatus* on Hematological Parameters

Treatment Groups	RBCs ($x10^6$/µl)	WBCs ($x10^3$/µl)	Platelets ($x10^3$/µl)	Hb (gm/dl)
Control	4.1±0.19	4.4±0.22	425±31.16	9.3±0.34
RCE-A (250 mg/kg)	4.2±0.2	4.2±0.21	375±15.16*	8.9±0.15
RCE-B (500 mg/kg)	3.9±0.17	5.2±0.21*	352±39.91**	9.9±0.55
RCE-C (1000 mg/kg)	4.1±0.14	7.2±0.18**	212±22.81**	9.1±0.26

n =10 per group, Values are Mean ± Std. Error of Mean, RCE: *Raphanus caudatus* Extract, *p ≤ 0.05 significant versus control, **p ≤ 0.005 highly significant versus control

It is fact that these ROS provide protection against pathogenic microorganisms, but it also causes tissue damage which ultimately lead to progression of inflammatory disorders which might be reason of various diseases (Johansen, Harris *et al*. 2005). Chemiluminescence assay is based on the luminescent capacity of luminol by the attacking of free radicals in the sample (Roginsky and Lissi 2005).

This study revealed that RCE significantly have immunomodulatory/anti-inflammatory effect against ROS in vitro testing by chemiluminescence assay. Activation of antioxidant enzymes and inhibitory potential against ROS has been studied in another study by Tunisian radish extract (Salah-Abbès *et al*. 2008). Lately Salah-Abbès and co-workers also stated about the protective effects of root extract of *Raphanus sativus* in 2010 while immunotoxicity was induced by high dose of zearalenone. Fruits and Vegetables are the rich source of alkaloids, saponin and phenols (Liu 2003 and Oz 2017) that have very important effect in inhibition of oxidation process that could be attributed to observed effects in RCE.

As we all know that inflammation is protective mechanism of body and in our current study when inflammation was induced by acetic acid induced rat paw edema model the results of table 3 findings showed that RCE have significant anti-inflammatory effect (Libby 2007). The inflammatory mediators majorly effect in inflammation are released in two stages.

Initially in two hours of inflammatory mediators like histamine and serotonin are released while in second stage prostaglandins, proteases and kinins are released after 3-4 hours of acetic acid injection which plays major role in inflammation (Lomba *et al*., 2017). After the dosing protocol all tested doses (250, 500 and 1000mg/kg) of RCE, we have found significant reduction in rat's paw while the highest percentage of paw edema inhibition 45.01% was observed at the dose of 1000 mg/kg which is greater than our standard drug acetylsalicylic acid at same time i.e. 33.06%. So it indicates that RCE may act particularly via inhibition of prostaglandin synthesis. Kim *et al*. (2015 and 2014) found in their studies that seeds of *Raphanus caudatus* have some anti- inflammatory derivatives i.e. phenylpropanoid sucrosides and methylthio-butanyl which were observed by the inhibition of nitric oxide during in vitro anti- inflammatory activity (Kim *et al*. 2014 and 2015). In the other study of water extract of *Raphanus caudatus* seeds alleviate intestinal inflammation in trinitrobenzenesulphonic acid and dextran sodium sulfate -induced colitis in rats. The occurrence of sinapic acid was attributed to this anti-inflammatory effect (Kook *et al*. 2014 and Cho *et al*., 2016).

The occurrence of flavonoids has been reported in the pods of radish (Takaya *et al*. ,2003, Beevi *et al*., 2012) similarly in our study the anti-inflammatory activity of RCE, this might be a reason of this effect and support the folk use of *Raphanus sativus* in these disorder (B Aggarwal, Prasad *et al*. 2011). Furthermore, Radish is a great source of vitamin C which is well reported in different studies of anti-inflammatory and antioxidant activity (Peters, Anderson *et al*. 2001, Padayatty, Katz *et al*. 2003).

This study provides evidence that that leukocytes level was increased significantly by RCE at the completion of 500 and 1000mg/kg. Leukocytes have major role in defensive system of body. Increased leukocyte count supports the immunosuppression and healing of wounds. Though elevated leukocytes are the markers of blood related diseases and long term cancer like leukemia. Since it may assumes that the rich amount of alkaloids, flavonoids, terpenoid and isothiocyante compounds in *Raphanus caudatus* indicates enhance leukocyte levels in the current study and it has been supported by the Thejass and Kuttan (2007**).**

This study of RCE showed a remarkable reduction in number of platelet in dose dependent manner after long-term administration. In the current study, the platelet inhibiting activity of Radish pods is an interesting outcome. As discussed previously that Radish pods have high amount of alkaloids and isothiocyanate compound, especially sulforaphene and sulforaphane which possess anti-platelet activity that has been reported in several studies (Chuang *et al*., 2013, Fuentes and Palomo 2014, Jayakumar *et al*., 2016). However sulforaphane anti-platelet activity is reported in different studies which is due to stimulation of adenylate cyclase, glycoprotein IIb/IIIa as well as thromboxane A2 (Jayakumar *et al*., 2013, Oh *et al*., 2013, Ku and Bae 2014). The antiplatelet activity occurs due to the presence of alkaloids which is already reported in different studies (Chen *et al*., 1996). Therefore, the occurrence of natural compounds in Radish pods might be attributed to its platelet inhibiting activity in this study. The antiplatelet activity of Radish pods could have a protective effect in coronary artery diseases and thrombotic illnesses and caution must be taken in the usage before surgery.

CONCLUSION

Based on the results obtained from this study, it is concluded that *Raphanus caudatus* processes significant pharmacological potential in inflammation and hematological parameters. The plant produced significant anti-inflammatory activity *in vitro* and *in vivo*. This might be attributed because of the presence of flavonoids and vitamin C. Moreover, a statistically significant raised level of leukocytes at 500 and 1000 mg/kg with a dose-

dependent reduction in platelets count was prominent. It is recommended to carry out further study to explore the mechanism of observed anti-inflammatory and hematological potential.

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