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Research Paper

Non-clinical evaluation of antioxidant and anti-inflammatory activity of ethanolic *Citrus maxima* peel extract

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ABSTRACT

The peel of *Citrus maxima* (CM) was studied for its anti-oxidant and anti-inflammatory activity by applying a number of non-clinical testing methods. Anti-oxidant tests were carried out by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging as well as, reducing power and total flavonoid content (TFC) methods, while anti-inflammatory tests were done by carrageenan-induced paw edema and cotton pellet granuloma tests. The results suggest that CM concentration-dependently scavenged DPPH radicals as well as, exhibited potential reducing power when compared with the standard, ascorbic acid. The TFC value was 0.31 mg/g of quercetin equivalent. In the anti-inflammatory tests, CM dose-dependently exhibited significant ($p < 0.05$) paw edema and pellet granuloma. The highest activity was observed at CM 300 mg/kg. In conclusion, the CM exhibited anti-oxidant and anti-inflammatory effects in the *in vitro* and *in vivo* test systems. The CM may be one of the potential sources of lead compounds with anti-oxidant and anti-inflammatory drugs.

Keywords: *Citrus maxima,* anti-oxidant, anti-inflammatory.

INTRODUCTION

The inflammatory response involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair which are aimed at host defense and usually activated in most disease conditions (Rajendran and Lakshmi, 2008). However, chronic inflammation may lead to several diseases, including hay fever, periodontitis, atherosclerosis, rheumatoid arthritis, and even cancer (for example, gallbladder carcinoma).

Reactive oxygen species (ROS) play an important role in the pathogenesis of inflammatory diseases. Anti-oxidants capable of scavenging ROS are expected to improve these disorders (Parasuraman et al., 2014).

It is evident that, the plants have provided humans with many of their essential needs, including life-saving pharmaceutical agents. Recently, the World Health Organization (WHO) estimated that 80% people worldwide rely on herbal medicines. Thus, these natural sources can be used directly or indirectly in the development of new 'leads' to combat various diseases.

Citrus maxima (Burm.) Merr. (Family: Rutaceae) is a low-branching, evergreen tree growing 5 to 10 m tall with occasional specimens up to 15 m. It is habituated in Thailand, however, grown in other tropical regions, such as Florida, Brazil, Malaysia, India and Bangladesh etc. the medicinal applications of leaves, flowers, fruits and seeds, including the treatment of coughs, fevers and gastric disorders was also discovered. The fruit is cardiotonic and refrigerant; useful in influenza, cough, catarrh and asthma. The rind is anthelmintic; useful in vomiting, griping of abdomen and diarrhoea. The leaves are useful in epilepsy, chorea and convulsive cough. An alcoholic extract of the leaves is evident in its moderate anti-bacterial activities, while the essential oil possesses antifungal properties. Cirantin (a known component of the peel) possesses anti-fertility activity (http://www.mpbd.info/plants/citrus-grandis.php).

This study aims to evaluate anti-oxidant and anti-

inflammatory effects of peel of *C. maxima* by applying various *in vitro* and *in vivo* non-clinical studies.

MATERIALS AND METHODS

Collection, identification and extraction of plant sample

The *C. maxima* fruit peel of the plant was collected from the Chittagong hill tracts, Bangladesh and identified by an expert in Bangladesh National Herbarium, Mirpur, Dhaka. The peel was then cut into small pieces and subjected for sundry 14 days. The dried materials were grounded into coarse powder and stored in an airtight amber color container. Thereafter, the powder materials were extracted with ethanol by applying maceration technique. After 14 days, the content was filtered using a sterilized cotton filter and then through Whatman filter paper (No. 1). Finally, the solvent was evaporated by a rotary evaporator.

Reagents and chemicals

The standards (ascorbic acid, indomethacin and chlorpheniramine HCl) were purchased from the Square Pharmaceuticals Ltd., Bangladesh, while the ethanol and other reagents and chemicals (analytical grade) were purchased from the Merk, India.

Screening for antioxidant activity

DPPH free radical scavenging assay

The free radical scavenging activity of the extract was determined by their ability to scavenge stable radical DPPH (Monjur-Al-Hossain et al., 2013). An aliquot of (50 µl) extract solution (31.25 - 500 µg/ml) was mixed with 3 ml of a DPPH solution (40 µg/ml) in ethanol. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbances of the mixtures were measured at 517 nm spectrophotometrically, while AA was used as positive control. The corresponding percentage of inhibitions was calculated using the equation:

% DHHP radical scavenging capacity = [1- (Abs_{sample} / $Abs_{control}$)] × 100%

Where: Abs_{sample} and $Abs_{control}$ stand for the absorbance of the sample and the control, respectively.

Determination of reducing power capacity

The reducing power of the crude extract was determined in terms of their capacity to reduce ferric ion to ferrous ion (Hazra et al., 2008). Different concentrations (31.25 – 500 µg/ml) of the extract (1 ml) was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1%), followed by incubation at 50°C in a water bath for 20 min. Thereafter, 2.5 ml of trichloro acetic acid (10%) was added to terminate the reaction. The upper portion of the solution (2.5 ml was mixed with 2.5 ml distilled water, and 0.5 ml $FeCl_3$ solution (0.1%). After keeping it at room temperature for 10 min, the absorbance was measured at 700 nm against an appropriate blank solution, while AA was used as standard.

Total flavonoid content (TFC)

Total flavonoid content (TFC) of the samples was determined using quercetin as standard (Hazra et al., 2008). The result of TFC was expressed as quercetin equivalent (mg QE/g dry matter), as calculated from the prepared standard curve.

Anti-inflammatory activity tests

Experimental animals

Long Evans rats (*Rattus norvigicus*) of either sex, weighing 100 to 150 g were provided by the animal house of Department of Pharmacy, NSU and used for the anti-inflammatory tests. All the animals were acclimatized one week prior to conducting of the experiments maintaining standard laboratory conditions (relative humidity: 55-65%, room temperature: 25.0 ± 2°C and 12 h light/dark cycle). The animals were fed with standard diet from the International Center for Diarrheal Diseases and Research, Bangladesh (ICDDR, B) and had free access to water *ad libitum*.

Carrageenan induced paw edema test

The rats were divided into 6 groups (n = 5) (NC, INDO, CHLOR and three groups for CM) randomly. Before treatment, a reading of paw volume at 0 h was recorded for each group. The negative control (NC) group treated with saline water (0.9% NaCl solution at 10 ml/kg, p.o.), while the INDO (indomethacin) and CHLOR (chlorpheniramine HCl) were given at 10 mg/kg (p.o.) and 2 mg/kg (i.p.), respectively. The CM (*C. maxima*) was administered at 100, 200 and 400 mg/kg (p.o.). After half an hour of sample/drug administration, 0.1 ml of 1% carrageenan solution was injected into the sub-plane surface of the right hind paw of each animal. Time of observation was 1 to 6 h. Paw volume (ml) was detected using a Plethysmometer (Winter et al., 1962).

Table 1: DPPH radical scavenging capacity.

Concentration (µg/ml)	% Scavenging of DPPH radicals	
	AA	CM
31.25	77.44 ± 0.19	66.74 ± 0.42
62.5	85.35 ± 0.88	72.09 ± 0.30
125	86.74 ± 0.42	78.60 ± 0.47
250	88.14 ± 0.95	81.86 ± 0.05
500	89.07 ± 0.98	85.81 ± 0.40
Other parameters		
IC_{50} (µg/ml)	8.854 ± 0.07	8.037 ± 0.13
CI (µg/ml)	5.175 - 15.15	3.215 - 20.09
R^2	0.99	0.96

Values are mean ± SEM (n = 5), One way Analysis of Variance (ANOVA) followed by Dunnett test considering $p < 0.05$; AA: ascorbic acid; CM: *Citrus maxima*; IC_{50}: half-minimal inhibitory concentration; CI: confidence of interval; R^2: coefficient of determination.

Cotton wool granuloma test

Animals were grouped randomly into 5 groups (n = 5) (NC, PRED, and three groups for CM). The negative control (NC) group treated with saline water (0.9% NaCl solution at 10 ml/kg, p.o.), while the PRED (prednisolone) was given at 10 mg/kg (i.p). The CM (*C. maxima*) was administered at 100, 200 and 400 mg/kg (p.o.), respectively. The animals were anesthetized 1 h after dosing by ketamin (75 mg/kg, i.p.). Thereafter, the furs of the axilla area were cleaned with the help of a scissor and razor. This area is wiped with 70% v/v ethanol. A small subcutaneous incision was made in the axilla region which formed a pouch using blunt ended forceps. Thereafter, 20 ± 1 mg of the sterile cotton pellet was inserted in the axilla. The incisions were sutured by sterile catgut/biodegradable surgical stings**.** The animals were then kept in separate cages until recovery from anesthesia and were returned to their home cages and fed with food and water *ad libitum*. The animals were treated with extract/controls once daily for 7 consecutive days from the day of cotton pellet insertion. On the 8th day, the animals were euthanized (by ketamin, 100 mg/kg, i.p.) and sacrificed and cotton pellets covered by the granulomaus tissue which were surgically removed. Pellets were separated from extraneous tissue and dried at 60°C until 24 h. The percent change of the granuloma weight inspect of NC group was determined for each group according to the method described by Panthong et al. (2003).

Statistical analysis

Results are presented as mean of standard error of mean (SEM). The data were analyzed by means of analysis of variance (ANOVA) followed by Dunnett's test considering $p < 0.05$ using GraphPad Prism (version 6.0) with a confidence level of 95%.

RESULTS

Antioxidant tests

CM concentration-dependently scavenged DPPH radicals, where highest percent of inhibitory capacity (85.81 ± 0.40) was seen with 500 µg/ml. However, the standard drug AA showed a better DPPH scavenging capacity than the CM. The IC_{50} values calculated for AA and CM were 8.854 ± 0.07 (CI: 5.175 - 15.15) and 8.037 ± 0.13 (CI: 3.215 - 20.09) µg/ml, respectively (Table 1).

Table 2 suggests that CM concentration-dependently exhibited reducing power capacity. The highest reducing power capacity was observed at 500 µg/ml of CM and AA. The IC_{50} values calculated for AA and CM were 33.37 ± 0.02 (CI: 28.99 - 38.40) and 58.40 ± 0.02 (CI: 52.03 - 65.55) µg/ml, respectively. The calculated TFC value was 0.31 mg/g of quercetin equivalent ($y = 0.0098x - 0.036$).

Anti-inflammatory tests

CM reduced carrageenan-induced paw edema in rats in a time and dose-dependent manner (with some exceptions). The highest reduction of paw edema was seen at 400 mg/kg at the 6th hour. The standard, INDO at 10 mg/kg also reduced paw edema time-dependently; however, the highest reduction was observed at the 6th hour (Table 3).

The granuloma reduction capacity when compared with the standard drug, PRED suggest that there is a dose-depended activity. However, the CM was found to reduce dry cotton granuloma significantly ($p < 0.05$) than the wet cotton granuloma in experimental animals (Table 4).

Pearson's correlations

According to the Figure 1, it is clear that the DPPH

Table 2: Reducing power capacity.

Concentration (µg/ml)	% Reducing power	
	AA	CM
31.25	45.26 ± 0.31	23.33 ± 0.27
62.5	73.70 ± 0.39	43.33 ± 0.91
125	86.74 ± 0.48	63.33 ± 0.67
250	91.56 ± 0.72	76.67 ± 0.72
500	97.41 ± 0.61	83.33 ± 0.34
Other parameters		
IC_{50} (µg/ml)	33.37 ± 0.02	58.40 ± 0.02
CI (µg/ml)	28.99 - 38.40	52.03 - 65.55
R^2	0.99	0.99

Values are mean ± SEM (n = 5), One way Analysis of Variance (ANOVA) followed by Dunnett test considering $p < 0.05$; AA: ascorbic acid; CM: *Citrus maxima*; IC_{50}: half-minimal inhibitory concentration; CI: confidence of interval; R^2: coefficient of determination.

Table 3: Anti-inflammatory test induced by carrageenan-induced paw edema in rats.

Treatments		% Inhibition of paw edema					
		1	2	3	4	5	6
		Hours (h)					
NC (saline water: 10 ml/kg)		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
INDO (10 mg/kg)		12.73 ± 0.41	21.65 ± 0.34	37.31 ± 0.19	34.83 ± 0.34	47.48 ± 0.45	50.12 ± 0.17
CHLOR (2 mg/kg)		7.43 ± 0.20	9.88 ± 0.34	13.93 ± 0.32	14.07 ± 0.52	10.24 ± 0.20	19.32 ± 0.32
CM (mg/kg)	100	0.79 ± 0.24	2.97 ± 0.35	3.46 ± 0.48	6.61 ± 0.30	7.06 ± 0.27	10.06 ± 0.20
	200	17.92 ± 0.17	8.49 ± 0.15	10.55 ± 0.28	18.68 ± 0.22	20.48 ± 0.25	28.06 ± 0.16
	400	23.35 ± 0.48	11.54 ± 0.47	33.47 ± 0.25	34.37 ± 0.26	35.89 ± 0.33	44.87 ± 0.17

Values are mean ± SEM (n = 5). ANOVA followed by *post hoc* analysis with a one-tailed Dunnett's t-test for multiple comparisons; NC: negative control, INDO: indomethacin, CHLOR: chlorpheniramine HCl and CM: *Citrus maxima*.

Table 4: Anti-inflammatory activity by cotton pellet granuloma test in rats.

Treated groups		Weight of cotton pellet	
		% Inhibition of wet cotton granuloma	% Inhibition of dry cotton granuloma
NC (saline water: 10 ml/kg)		0.00 ± 0.00	0.00 ± 0.00
PRED (10 mg/kg)		42.30 ± 2.24	41.50 ± 1.80
CM (mg/kg)	100	20.72 ± 3.29	14.20 ± 2.25
	200	31.14 ± 1.12	32.20 ± 2.07
	400	37.34 ± 3.24	38.93 ± 2.59

Values are mean ± SEM (n = 5), ANOVA followed by *post hoc* analysis with a one-tailed Dunnett's t-test for multiple comparisons; NC: negative control, PRED: prednisolone, CM: *Citrus maxima*.

scavenging capacity is significantly related to reducing power capacity, while dry cotton granuloma is related to the reduction of wet cotton granuloma.

Figure 2 is suggests a relationship between the antioxidant and anti-inflammatory activity of the CM. An increase in radical scavenging or reducing power capacity of the CM is directly proportional to the reduction of paw edema in rats.

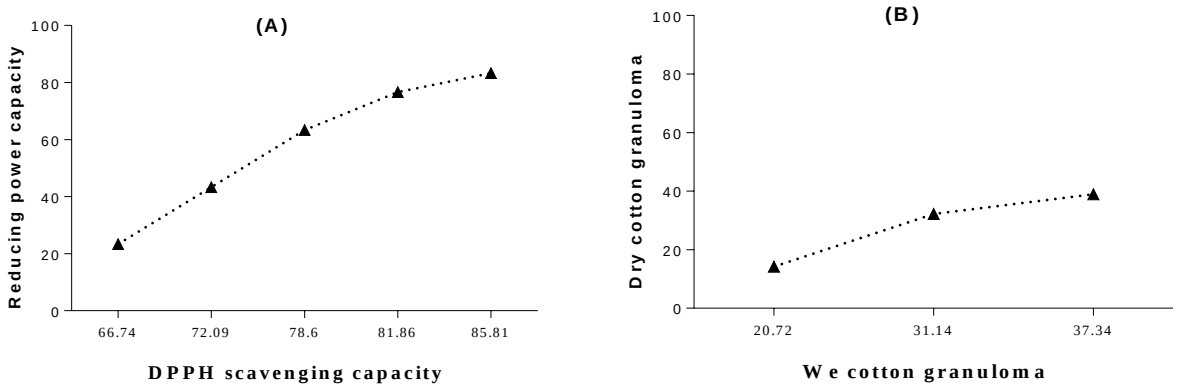

Figure 1: Pearson's correlation between (A) DPPH radical scavenging vs. Reducing power capacity, and (B) Dry cotton granuloma vs. Wet cotton granuloma.

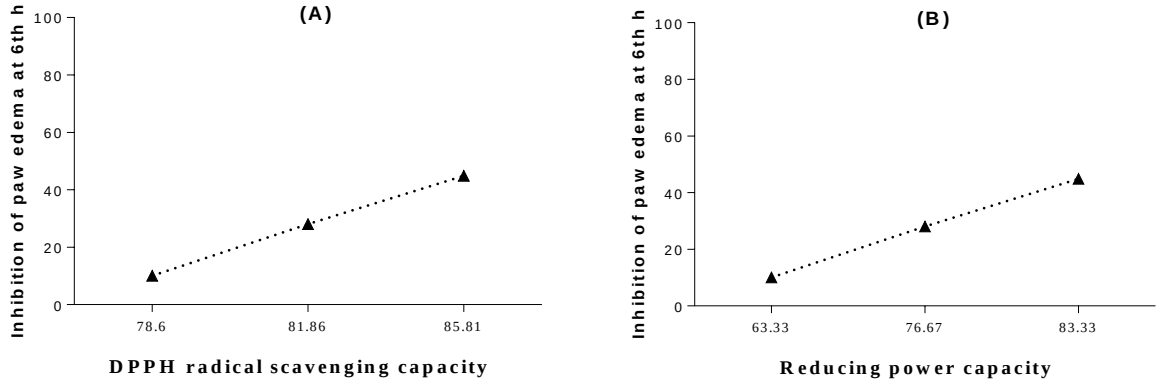

Figure 2: Pearson's correlation between (A) DPPH radical scavenging vs. inhibition of paw edema at 6th h, and (B) Reducing power capacity vs. inhibition of paw edema at 6th h in rats.

The DPPH scavenging activity and reducing power capacity of CM at 125, 250 and 500 µg/ml when compared with the granuloma reducing capacity in rats found a good correlation (Figure 3).

DISCUSSION

It is well established that consumption of foods containing phytochemicals with potent antioxidant activity can reduce the progression of chronic diseases associated with oxidative stress (Khan et al., 2016). Apart from providing basic nutrition, most vegetables and fruits serve as functional foods by virtue of their antioxidant capacity (Kaur and Kapoor, 2001). Excess generation of free radicals within the living systems can cause serious damage to tissues and different organs, leading to life threatening diseases (Rahman et al., 2015). The free radical scavenging potential of the CM peel extract was determined on the basis of their scavenging ability of the stable DPPH free radical, while reducing power through the ability to reduce the ferric ions (Fe^{3+}) to ferrous (Fe^{2+}). The reducing capacity of a substance contributes towards its antioxidant potential along with the factors like chain initiation, decomposition of peroxides, reducing capacity and radical scavenging (Hazra et al., 2008). Our study also demonstrates a Pearson's correlation between the DPPH radical scavenging activity and reducing power capacity of CM.

It is also believed that the localization of flavonoids within the artificial and biological membranes and interactions of flavonoids at the surface of lipid bilayer can reduce the access of deleterious molecules (that is, oxidants) and protect the structure and function of membranes from oxidants (Oteiza et al., 2005). Therefore, the TFC of the investigated medicinal plant was of interest when evaluating their antioxidant properties. The low TFC value of CM indicates possible other potent antioxidant and anti-inflammatory components present in the peel of CM.

Carrageenan induced rat paw edema is commonly used as an experimental animal model for assessment of acute inflammation and is believed to be bi-phasic, of which the first phase is mediated by the release of histamine and 5-HT in the early stage followed by release of kinin and then prostaglandin and bradykinins in the later phase (Mazumder et al., 2003). From the present results it is

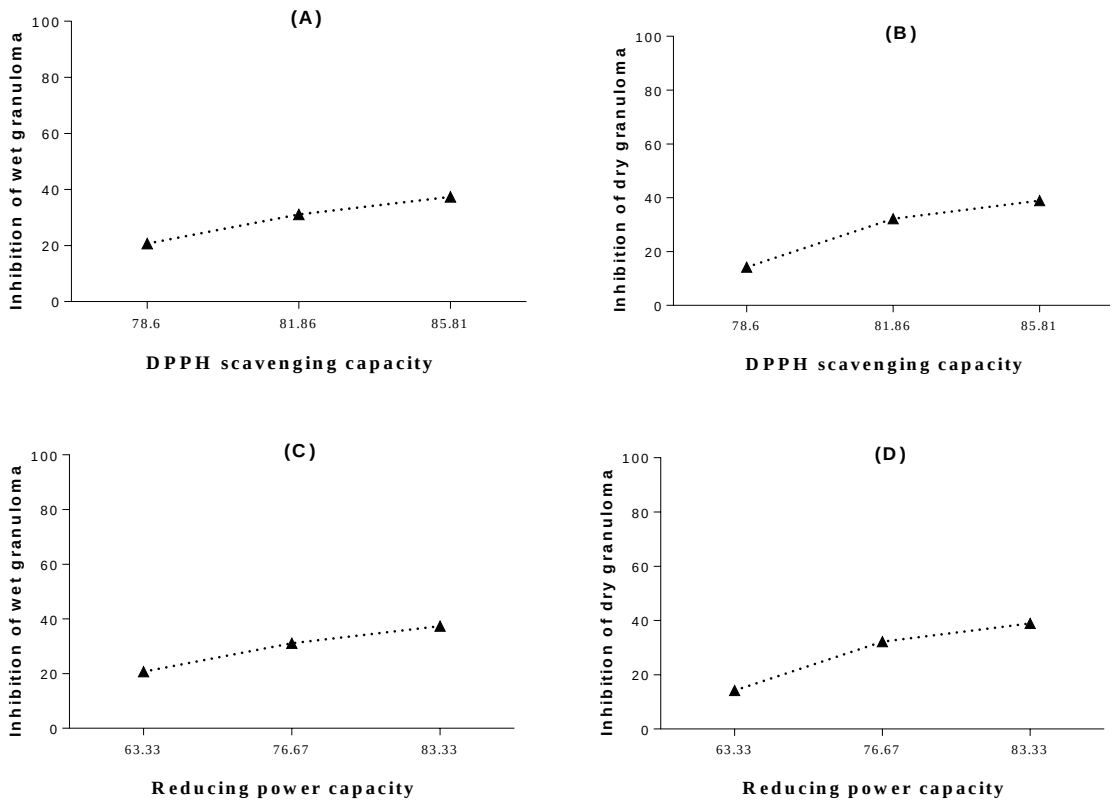

Figure 3: Pearson's correlation between (A) DPPH radical scavenging *vs* Inhibition of wet cotton granuloma, (B) DPPH radical scavenging vs. inhibition of dry cotton granuloma, (C) Reducing power capacity vs. inhibition of wet cotton granuloma, and (D) Reducing power capacity vs. inhibition of dry cotton granuloma.

evident that ethanol extract of CM exhibited maximum activity at dose 400 mg/kg and the effect was significant ($p < 0.05$) in comparison to the standard.

On the other hand, the cotton wool granuloma test is widely used to evaluate the transudative and proliferative components of chronic inflammation. The weight of the wool correlates with transudes, while the dry weight of the wool correlates with the amount of granulumatous tissues. Chronic inflammation occurs by means of the development of proliferating cells. These cells can spread in granuloma form. Non-steroidal anti-inflammatory drugs decrease the size of granuloma which results from the cellular reaction by inhibiting granulocyte infiltration, preventing the generation of collagen fibers and suppressing muco-polysaccharides (Ashok et al., 2010). The CM showed significant ($p < 0.05$) anti-inflammatory activity in both studies, thus, may be effective in the chronic inflammatory condition, which reflects its efficacy in inhibiting an increase in the number of fibroblasts and synthesis of collagen and muco-polysaccharides during granuloma tissue formation.

ROS play an important role in the pathogenesis of inflammatory diseases. Antioxidants are capable of scavenging ROS and act against these disorders (Parasuraman et al., 2014). Pearson's correlation between the antioxidant activity and anti-inflammatory effects of CM suggest an interrelationship.

Conclusion

The peel of *C. maxima* (CM) concentration/dose-dependently exerted anti-oxidant and anti-inflammatory effects in the test systems. ROS scavenging and potential reducing power capacity of CM may be linked to its anti-inflammatory effects in test animals. Further research is necessary to isolate, characterize and determine mechanisms for each pharmacological activity.

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