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Anti-inflammatory and analgesic activities of Tunisian *Citrullus colocynthis* Schrad. immature fruit and seed organic extracts

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Abstract. – *Background and Objectives:* Inflammations and immune-related diseases including rheumatoid arthritis are widespread in the entire globe. The treatment of these illnesses is mainly based on the use of synthetic and biotechnological drugs, in recent years. Tunisian traditional medicine is a potential source of new remedies namely *Citrullus colocynthis* Schrad. (Cucurbitaceae): endemic in southern Tunisia and used in folk medicine to treat many inflammation disorders. Our goal was to assess the *in vivo* analgesic and anti-inflammatory activities of Tunisian *Citrullus colocynthis* immature fruit and seed organic extracts (petroleum ether, chloroform, ethyl acetate, acetone and finely methanol extract).

***Material and Methods:* Yields of prepared organic extracts are gravimetrically determined. For the analgesic and anti-inflammatory activities, we have used respectively, the acetic acid writhing test in mice and the carrageenan-induced paw edema assay in rats.**

***Results and Discussion:* All extracts displayed an important analgesic and anti-inflammatory activities at different doses without inducing any side effects. This study has demonstrated the analgesic and anti-inflammatory activities of *Citrullus colocynthis* immature fruit and seed extracts. Experiment results provide scientific insight into the ancient practice of utilizing *Citrullus colocynthis* Schrad. as analgesic and as anti-inflammatory agents.**

Key Words:

Citrullus colocynthis Schrad., Fruits, Seeds, Anti-inflammatory, Analgesic.

Introduction

Since ancient times, several diseases have been treated by administration of plant extracts. Interest in ethnopharmacy as a source of bioactive compounds has increased worldwide, particularly in the search for anti-inflammatory drugs. Plants with analgesic and anti-inflammatory activities have become more interesting because some of them are part of the arsenal of modern medicine and many people are aware of problems associated with the over-prescription and misuse of usual drugs.

Citrullus colocynthis Schrad., belonging to the family of Cucurbitaceae and growing in arid areas, is endemic in the South of Tunisia[1]. This medicinal plant popularly known as Handhal, Hdaj or Dellaa El-Wad, is widely used in Tunisian folk medicine for treating many diseases such as hypertension and inflammation diseases, including rheumatism and rheumatoid arthritis[2,3].

Anti-inflammatory traditional healers seem to not pay attention to the fruit's degree of maturity and the literature rarely mentions if seeds are present in preparations involving ground fruit/pulp. Common preparations use juice, fresh or dried (often ground) fruit materials. Extracts (maceration or boiling) are prepared either in water or in aqueous mixtures (honey, milk, water/olive oil at various ratios). Methods of administration are by ingestion or massage[2,4-6].

Some studies have demonstrated the medicinal effect of *Citrullus colocynthis* Schrad. as anti-tu-

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mour[7], immunostimulant[8], anti-microbial[9,10], antioxidant[11] and against hepatic diseases[12], hyperglycaemia[13] and hair loss[14].

In our previously study we have demonstrated that the plant organ aqueous extracts with the highest analgesic and anti-inflammatory properties were from immature fruits and seeds. The most active organs of all were immature fruits[15]. The current study carried out a more exhaustive analysis of reproductive organs of *Citrullus colocynthis* Schrad. from south Tunisia. *In vivo* testing of analgesic and anti-inflammatory activities were carried out on organic extracts from immature seeds and fruits, using mice and rats as models.

Material and Methods

Plant Materials

Citrullus colocynthis Schrad. plants were collected in August 2007 near Medenine, Tunisia in the municipality of Sidi Makhlouf (33°33N, 10°27W). The identification was performed according to the flora of Tunisia[1] (Pottier-Alapetite, 1981) and a voucher specimen (C.C-01.01) deposited in the Biological Laboratory of the Faculty of Pharmacy of Monastir.

Chemical and Drugs

The organic solvents used, purchased from Sigma, were 99% pure. Carrageenan (BDH Chemicals Ltd Poole, England) and acetyl salicylate of lysine (ASL) were from Adwya Laboratory, Tunisia.

Extraction Protocol

Different solvents, petroleum ether, chloroform, ethyl acetate, acetone and methanol in ascending polarity, were used for Soxhlet extraction to fractionate the soluble compounds from the grape pomace. The extraction was performed with dried powder placed inside a thimble made by thick filter paper, loaded into the main chamber of the Soxhlet extractor, which consisted of an extracting tube, a glass balloon and a condenser. The total extracting time was 6 h for each solvent continuously refluxing over the sample (grape pomace). The resulting extracts were evaporated at reduced pressure to obtain the crude extracts.

Animals

Male adult Wistar rats weighing 160-180 g and Swiss albinos mice (weighing 18-25 g) of both sex were obtained from Pasteur institute (Tunis, Tunisia). They were housed in polypropylene cages and were left for 2 days for acclimatization to animal room maintained under controlled conditions (a 12 h light-dark cycle at 22 ± 2°C) on standard pellet diet and water ad *libitum*. Before the day of assay, only the Wistar rats were fasted overnight with the free access to water. Housing conditions and *in vivo* experiments approved according to the guidelines established by the European Union on Animal Care ([Council (86/609)]. The rats were used for the anti-inflammatory evaluation of the *Citrullus colocynthis* seed and fruit extracts while the mice were used for the analgesic investigation. Animals were divided into drug-treated "test" and saline-treated "control" groups of six or eight animals per group.

Analgesic Activity

Analgesic activity was performed according to the method of Koster et al.[16] and assessed by the acetic acid abdominal constriction test (writhing test) – a chemical visceral pain model. Swiss albino mice were selected one day prior to each test and were divided into groups of six mice each. One group served as the control and was pretreated under cutaneously with 10 ml/kg of saline. Another group was pretreated with the reference drug, Acetyl Salicylate of lysine (ASL), 200 mg/kg, by the same route. The remaining groups were injected intraperitoneally (i.p.) with 10 ml/kg of 1% acetic acid solution 30 min after the administration of different extracts at the doses of 0.05, 0.1, 0.5 and 2 mg/kg. After acetic acid administration, the number of writhes was counted during 30 minutes. Antinociceptive activity was expressed as inhibition percent of the usual number of writhes observed in control animals. The percentages of inhibition were calculated according to the following formula:

$$\% \text{ inhibition} = \frac{(\text{Number of writhes})_{\text{control}} - (\text{Number of writhes})_{\text{treated group}}}{(\text{Number of writhes})_{\text{control}}} \times 100$$

Anti-Inflammatory Activity

The anti-inflammatory activity was assessed on the basis of inhibition of paw edema induced

by the injection of carrageenan (an edematogenic agent) into the subplantar region of the right hind paw of the rat[17]. Male Wistar rats were divided into different groups of eight animals. The control group received 2.5 ml/kg of saline, the standard group received the reference drug (acetyl salicylate of lysine (ASL), 300 mg/kg) and the test groups received different extracts of *Citrullus colocynthis* at a dose of 0.5 and 1 mg/kg. Thirteen minutes after intraperitoneal administration of different substances, 0.05 ml of 1% of carrageenan suspension was injected to all animals in the right hind paw.

The paw volume, up to tibiotarsal articulation, was measured using a plethysmometer. The measures were determined at 0 h (V_0: before edematogenic agent injection) and 1, 2, 3, 4, 5, 6, 24 h intervals later (V_T). The difference between V_T (1, 2, 3, 4, 5, 6 and 24 h) and V_0 was taken as the edema value. The percentages of inhibition were calculated according to the following formula: % inhibition = $[(V_T\text{-}V_0)_{control} - (V_T\text{-}V_0)_{treated\ group}] \times 100 / (V_T\text{-}V_0)_{control}$

Statistical Analysis

Data obtained from animal experiments were expressed as means ± SE and as percentage. Results were statistically evaluated by ANOVA and using Student's *t*-test. $p \leq 0.05$ were considered significant.

Results

Extraction Yields

Yields of prepared extracts are given in Table I. Petroleum ether extraction gave the worse yield for fruits and the better one for seeds. For this last organ, the lowest yield is obtained for chloroform extraction. The higher yield of all is noted for fruit methanol extract.

Analgesic Activity

The inhibition percentages of writhing for all extracts are shown in Table II and Table III. The reference drug inhibited 61.88% of the number of writhing elicited by acetic acid. The analgesic effect was tested for concentrations ranging from 0.05 to 1 mg/kg. The administration of all tested extracts induced a potent dose-dependent antinociceptive activity without provoking any side effects. The strongest activities were obtained with polar immature seed and fruit extracts (acetone and methanol extracts). These extracts (either from seeds or from fruits) demonstrated a close property. Regarding the both organs, the analgesic effect increase with the extract polarity (seeds: 91.56% (chloroform extract) – 95.68% (methanol extract); fruits: 82.96% (petroleum ether extract) – 95.739% (methanol extract)) excepting petroleum ether seed extract (93.42%).

Anti-Inflammatory Effect

In carrageenan-induced rat paw edema, all seed and fruit extracts, without inducing any side effects, inhibited hind paw edema throughout the entire period of observation (Tables IV and V). This inhibition differs with the plant organ and its extract type. For this more exhaustive analysis of the anti-inflammatory activity of *Citrullus colocynthis* seeds and fruits, the obtained results demonstrate that the reduction of the paw edema vary in a dose dependent fashion with a maximum attend at 1 mg/kg. On the contrary, at 0.5 mg/kg, only an appreciably activity was noted. With all extracts, three hours after carrageenan injection, the anti-inflammatory activity instigate unambiguous increasingly to attend the maximum at 6 h and 24 h. In terms of plant organs, seeds and fruits showed a high significant activity from the control. The inhibition percentages were ranged, respectively at 6 and 24 h, from 75.06 to 96.00% and from 87.78 to 98.07% for immature fruits, and ranged from 83.53 to 93.88% and from 85.21 to 99.04% for immature

Table I. Yields (%) of *Citrullus colocynthis* seed and fruit extracts.

	P.E.		Chl.		E.A.		A.		M.	
	Seeds	Fruits	Seeds	Fruits	Seeds	Fruits	Seeds	Fruits	Seeds	Fruits
Yields	8.96	0.54	0.71	8.35	0.08	0.93	4.74	6.55	6.83	18.02

P.E.: petroleum ether extract; Chl.: chloroform extract; E.A.: ethyl acetate extract; A: Acetone extract; M.: Methanol extract.

Table II. Analgesic activity of *Citrullus colocynthis* Schrad. fruit extracts and reference drug (ASL).

Fruit extracts	Dose (mg/kg)	Number of writhes	Inhibition of writhing (%)
Control	–	74.33 ± 4.64	–
Petroleum ether extract	0.05	25.5 ± 0.54*	65.70
	0.1	24.17 ± 0.75*	67.49
	0.5	22.67 ± 0.82*	69.51
	1	12.67 ± 0.82*	82.96
Chloroform extract	0.05	15.33 ± 1.03*	79.37
	0.1	10.33 ± 0.52*	86.10
	0.5	9.50 ± 0.55*	87.22
	1	8.00 ± 0.89*	89.24
Ethyl acetate extract	0.05	9.67 ± 0.82*	86.99
	0.1	7.83 ± 0.75*	89.46
	0.5	6.50 ± 0.84*	91.26
	1	5.33 ± 0.82*	92.83
Acetone extract	0.05	8.33 ± 0.82*	88.79
	0.1	7.50 ± 0.55*	89.91
	0.5	3.66 ± 0.82*	95.07
	1	3.50 ± 0.84*	95.29
Methanol extract	0.05	7.16 ± 0.75*	90.36
	0.1	6.33 ± 0.52*	91.48
	0.5	5.00 ± 0.89*	93.27
	1	3.16 ± 0.98*	95.74
Reference drug (ASL, 200 mg/Kg)		28.33 ± 2.06*	61.88

Values are expressed as means ± SE (N=6); *≤ 0.001 significant from control; ASL: Acetyl Salicylate of Lysine.

Table III. Analgesic activity of *Citrullus colocynthis* Schrad. seed extracts and reference drug (ASL).

Seed extracts	Dose (mg/kg)	Number of writhes	Inhibition of writhing (%)
Control	–	81.00 ± 0.82	–
Petroleum ether extract	0.05	22.5 ± 1.05*	72.22
	0.1	14.00 ± 0.89*	82.72
	0.5	8.66 ± 0.82*	89.30
	1	5.33 ± 0.82*	93.42
Chloroform extract	0.05	23.00 ± 0.89*	71.60
	0.1	15.00 ± 0.89*	81.48
	0.5	11.50 ± 1.05*	85.80
	1	6.83 ± 0.98*	91.56
Ethyl acetate extract	0.05	11.50 ± 1.05*	85.80
	0.1	8.50 ± 0.55*	89.51
	0.5	6.83 ± 0.75*	91.56
	1	6.50 ± 0.84*	91.98
Acetone extract	0.05	11.50 ± 0.55*	85.80
	0.1	8.50 ± 1.05*	89.51
	0.5	5.00 ± 0.89*	93.83
	1	3.83 ± 0.75*	95.27
Methanol extract	0.05	10.00 ± 0.89*	87.65
	0.1	8.00 ± 0.89*	90.12
	0.5	6.50 ± 0.84*	91.97
	1	3.50 ± 0.55*	95.68
Reference drug (ASL, 200 mg/Kg)		28.33 ± 2.06*	61.88

Values are expressed as means ± SE (N=6); *≤ 0.001 significant from control; ASL: Acetyl Salicylate of Lysine.

Table IV. Effects of different *Citrullus colocynthis* Schrad. fruit extracts and reference drug on carrageenan-induced paw edema.

Extract	Dose (mg/Kg)	Mean swelling thickness (10^{-2}) ± S.E.M. (% inhibition)						
		1 h	2 h	3 h	4 h	5 h	6 h	24 h
Control 1	–	33.00 ± 1.76	48.00 ± 2.66	61.50 ± 4.77	76.25 ± 5.56	95.00 ± 5.25	106.25 ± 8.20	77.75 ± 3.51
Immature fruits								
Petroleum ether extract	0.5	22.50 ± 1.91*** (31.82)	26.75 ± 0.96*** (44.27)	33.25 ± 1.71*** (45.93)	38.50 ± 1.29*** (49.51)	34.5 ± 1.29*** (63.68)	37.75 ± 1.71*** (64.47)	19.00 ± 1.41*** (75.56)
	1	15.25 ± 0.96*** (53.79)	21.50 ± 1.29*** (55.21)	24.50 ± 1.29*** (60.16)	22.00 ± 1.82*** (71.15)	15.5 ± 0.58*** (83.68)	12.75 ± 0.96*** (88.00)	9.25 ± 0.50*** (88.10)
Chloroform extract	0.5	22.00 ± 0.82*** (33.33)	29.50 ± 1.91*** (38.54)	33.00 ± 1.41*** (46.34)	41.00 ± 0.82*** (46.23)	33.00 ± 2.58*** (65.26)	30.00 ± 1.41*** (71.76)	20.25 ± 1.71*** (73.95)
	1	16.50 ± 1.91*** (50.00)	22.00 ± 1.83*** (54.16)	25.50 ± 1.29*** (58.54)	15.75 ± 2.28*** (79.34)	14.75 ± 0.58*** (84.47)	16.25 ± 0.96*** (84.71)	5.00 ± 0.82*** (93.57)
Ethyl acetate extract	0.5	25.75 ± 0.96*** (21.97)	35.00 ± 2.16*** (27.08)	38.00 ± 0.82*** (38.21)	41.75 ± 1.26*** (45.25)	35.25 ± 1.26*** (62.89)	40.00 ± 0.82*** (62.35)	16.50 ± 1.00*** (78.78)
	1	24.50 ± 1.29*** (25.76)	31.75 ± 1.71*** (33.85)	28.00 ± 1.86*** (54.47)	30.50 ± 1.29*** (60.00)	25.50 ± 1.29*** (73.16)	26.50 ± 0.58*** (75.06)	9.50 ± 0.58*** (87.78)
Acetone extract	0.5	24.75 ± 1.50*** (25.00)	32.25 ± 1.71*** (32.81)	34.75 ± 0.96*** (43.50)	41.50 ± 1.29*** (45.57)	35.00 ± 0.82*** (63.16)	37.00 ± 2.58*** (65.17)	19.25 ± 0.96*** (75.24)
	1	20.25 ± 0.50*** (38.64)	18.00 ± 1.25*** (62.50)	15.25 ± 0.50*** (75.20)	11.00 ± 0.82*** (85.57)	6.45 ± 0.50*** (92.89)	4.25 ± 0.50*** (96.00)	1.50 ± 0.58*** (98.07)
Methanol extract	0.5	29.25 ± 1.71*** (11.36)	32.00 ± 0.82*** (33.33)	40.50 ± 1.29*** (34.15)	43.25 ± 3.20*** (43.28)	53.00 ± 2.45*** (44.21)	38.25 ± 0.96*** (64.00)	23.00 ± 1.15*** (70.42)
	1	15.25 ± 2.5*** (53.79)	20.75 ± 0.96*** (56.77)	25.00 ± 0.82*** (59.35)	26.50 ± 1.29*** (65.25)	30.50 ± 1.73*** (67.89)	11.50 ± 1.29*** (89.17)	5.00 ± 0.82*** (93.57)
Control 2	–	15.00 ± 1.76	34.50 ± 4.33	57.00 ± 6.22	61.50 ± 6.42	67.00 ± 7.37	71.00 ± 6.39	40.50 ± 5.84
ASL	300	7.25 ± 0.96*** (51.66)	14.25 ± 1.71*** (58.69)	17.50 ± 2.38*** (69.30)	15.75 ± 2.22*** (74.40)	19.50 ± 2.08*** (70.89)	19.50 ± 1.29*** (72.53)	23.75 ± 1.71*** (41.36)

Values are expressed as means ± SE (N=8); *$p \leq 0.05$, **≤0.01, ***≤0.001 significant from the control; ns: not significant from the control; ASL: Acetyl Salicylate of Lysine.

Table V. Effects of different *Citrullus colocynthis* Schrad. seed extracts and reference drug on carrageenan-induced paw edema.

Extract	Dose (mg/Kg)	Mean swelling thickness (10^{-2}) ± S.E.M. (% inhibition)						
		1 h	2 h	3 h	4 h	5 h	6 h	24 h
Control 1	-	33.00±1.76	48.00±2.66	61.50±4.77	76.25±5.56	95.00±5.25	106.25±8.20	77.75±3.51
Immature seeds								
Petroleum ether extract	0.5	18.25±1.71*** (44.70)	21.50±1.73*** (55.21)	22.50±2.08*** (63.41)	28.50±1.29*** (62.62)	31.00±2.83*** (67.37)	24.75±1.26*** (76.71)	3.00±0.82*** (96.14)
	1	17.25±0.96*** (47.72)	14.75±2.75*** (69.27)	16.00±1.41*** (73.98)	19.00±1.82*** (75.08)	17.75±1.71*** (81.32)	6.50±0.60*** (93.88)	0.75±0.50*** (99.04)
Chloroform extract	0.5	16.00±1.83*** (51.52)	25.50±1.91*** (46.04)	32.00±1.41*** (47.97)	39.00±2.45*** (48.85)	36.00±2.71*** (62.11)	28.75±0.96*** (72.94)	13.25±1.71*** (82.96)
	1	13.75±0.96*** (58.33)	19.50±0.58*** (59.38)	16.50±1.29*** (73.17)	9.00±1.55*** (88.20)	11.25±0.96*** (88.16)	6.50±0.58*** (93.88)	4.50±0.58*** (94.21)
Ethyl acetate extract	0.5	27.75±1.71*** (15.91)	29.50±1.29*** (38.54)	28.00±1.15*** (54.47)	31.25±0.96*** (59.02)	30.50±0.58*** (67.89)	23.75±0.96*** (77.65)	17.00±0.82*** (78.14)
	1	25.50±1.29*** (22.73)	27.25±0.96*** (43.23)	19.50±0.58*** (68.29)	17.50±1.29*** (77.05)	25.25±0.50*** (73.42)	17.50±1.29*** (83.53)	7.00±0.82*** (91.00)
Acetone extract	0.5	18.25±1.71*** (44.70)	22.50±1.29*** (53.13)	26.00±0.82*** (57.72)	25.75±1.71*** (66.23)	21.50±0.82*** (77.37)	21.75±1.71*** (79.53)	15.75±1.26*** (79.74)
	1	13.25±0.96*** (59.85)	19.25±0.96*** (59.89)	17.50±0.58*** (71.54)	20.00±2.16*** (73.77)	9.50±1.29*** (90.00)	7.75±0.96*** (92.71)	5.50±0.58*** (92.93)
Methanol extract	0.5	26.25±0.98*** (20.45)	37.50±0.58*** (39.02)	29.25±1.26*** (39.06)	31.25±0.98*** (59.02)	32.75±1.50*** (65.53)	25.75±0.96*** (75.76)	18.25±0.96*** (76.53)
	1	24.50±1.29*** (25.76)	22.25±0.96*** (53.65)	27.00±0.82*** (56.10)	25.50±0.58*** (66.56)	14.00±0.82*** (85.26)	15.75±0.96*** (85.18)	11.50±0.58*** (85.21)
Control 2	-	15.00±1.76	34.50±4.33	57.00±6.22	61.50±6.42	67.00±7.37	71.00±6.39	40.50±5.84
ASL	300	7.25±0.96*** (51.66)	14.25±1.71*** (58.69)	17.50±2.38*** (69.30)	15.75±2.22*** (74.40)	19.50±2.08*** (70.89)	19.50±1.29*** (72.53)	23.75±1.71*** (41.36)

Values are expressed as means ± SE (N=8); *$p \leq 0.05$, **≤0.01, ***≤0.001 significant from the control; ns: not significant from the controls; ASL: Acetyl Salicylate of Lysine.

seeds. Concerning the colocynth seeds, the highest activity was found for petroleum ether extract. Regarding the fruit activity, this extract type exhibited the less anti-inflammatory effect. Standard drug decreased paw edema by a maximum of 74.40% after 4 h.

Discussion

Compared with the previous report[15], and as a continuation of our study, this is the first exhaustive investigation evaluating the *in vivo* antinociptive and anti-inflammatory activities of Tunisian *Citrullus colocynthis* immature fruit and seed organic extracts collected from Medenine.

In acetic acid-induced writhing in mice, all tested samples extracts inhibited significantly writhing compared to controls. The obtained results support the hypothesis of *Citrullus colocynthis* participation in the inhibition of endogenous substances release including serotonin, histamine, prostaglandin and bradykinin[18] and arachidonic acid metabolites via cyclooxygenase[19]. The obtained peripheral analgesic property of seed and fruit organic extracts is probably linked to their anti-inflammatory effects.

Carrageenan has been widely used as a noxious agent able to induce experimental inflammation for the screening of compounds possessing anti-inflammatory activity. This phlogistic agent, when injected locally into the rat paw, produced a severe inflammatory reaction, which was discernible within 30 min[20]. The model of carrageenan-induced edema, according to Olajide et al.[21], involves two distinct phases: the early event (90-180 min) of the inflammation is due to the release of histamine, serotonin and similar substances. The later phase (270-360 min) is associated with the activation of kinin-like substances and the release of prostaglandins, proteases and lysosome. However, results were different for each organ extract depending on the early/later phases. The studied organic extracts inhibited the both phases of the carrageenan-induced edema by reducing the release of histamine and serotonin and also the kinin-like substances and prostaglandins. This pharmacological property may be attributed to the organ chemical composition: alkaloids, iridoids an steroids in fruits and seeds and flavonoids only in seeds[9] and to a possible molecular mechanism by effectively decreasing the production of the pro-inflammatory cytokines of IL-6 and IL-1β and the expression of COX-2 and simultaneously elevating the level of anti-inflammatory cytokine IL-4 in the carrageenan-injected rat paw tissues[22].

According to the results obtained in the present study, *Citrullus colocynthis* Schrad seems to interfere rather with the histamine and serotonin pathway and intensely with the prostaglandin and kinin-like pathway. This fact, taken together with the inhibition of abdominal writhing induced by acetic acid, strongly suggests that the organic extracts from *Citrullus colocynthis* Schrad. fruits. and seeds exert anti-inflammatory and analgesic properties and could be considered as effective agents to treat inflammation diseases. In fact, this plant, namely its non polar seed extracts and polar fruit extracts, demonstrated a high activity at very low doses (0.5 mg/kg and 1 mg/kg). The results presented here justified and supported scientifically its ethnopharmacological use and indicated that the natural products analyzed seem to be a good choice for the development of new strategies to treat pain and rheumatoid arthritis. Further studies are ongoing to identify and purify the chemical compounds of these analgesic and anti-inflammatory extracts.

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