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Behavioral and Biochemical Studies of Dichloromethane Fraction From the *Areca catechu* Nut

AHSANA DAR AND SHAGUFTA KHATOON

Pharmacology Section, H.E.J. Research Institute of Chemistry, International Center for Chemical Sciences, University of Karachi, Karachi-75270, Pakistan

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DAR, A. AND S. KHATOON. *Behavioral and biochemical studies of dichloromethane fraction from the* Areca catechu *nut.* PHARMACOL BIOCHEM BEHAV **65**(1) 1–6, 2000.—The dichloromethane fraction from *Areca catechu* was found to inhibit monoamine oxidase type A isolated from the rat brain with an IC_{50} of 665 ± 65.1 μg/ml. Studies with pharmacological models of depression, i.e., forced swim and tail-suspension tests, indicated that it caused significant reduction in the immobility time similar to that of moclobemide (a selective inhibitor of MAO-A) without causing a significant change in motor performance. Alkaloids such as arecaidine, arecoline, and a few other constituents, reported to be present in *Areca catechu* were also tested, but none of them were found to inhibit MAO. Present study suggests that the dichloromethane fraction from *A. catechu* possesses antidepressant property via MAO-A inhibition. © 1999 Elsevier Science Inc.

Areca catechu Dichloromethane fraction Antidepressant Synaptosomes 5-Hydroxytryptamine Monoamine oxidase-A

FOR ages, people living in different parts of the world have been using *Areca* nuts (*Areca catechu*) for masticatory purpose, and it is a very popular chewing nut in the Indo-Pak subcontinent. It has previously been reported that the nut has psychoactive (16,23) and antidepressant (11–13) effects. It is well established that animal models of depression such as forced-swim and tail-suspension tests are useful tools for the assessment of potential antidepressants (5,31). The duration of the immobility was reduced by a variety of antidepressants agents viz. imipramine, nialamide, and viloxazine (27), as well as aqueous ethanol extract, hexane, ethyl acetate, and aqueous fractions from *A. catechu* (11–13).

Drugs that are effective as antidepressants increase the availability of monoamines, 5-hydroxytryptamine (5-HT), or noradrenaline (NA). This happens either by preventing its enzymatic breakdown, as in the case of monoamine oxidase inhibitors (MAOIs), or by preventing its reuptake, as in the case of tricylic antidepressants and selective inhibitors of 5-HT reuptake (4,20).

Monoamine oxidase [MAO, EC 1. 4. 3. 4] causes oxidative deamination of biogenic amine and xenobiotics, and regulates the intracellular concentration of catecholamines and 5-HT in the brain and peripheral tissues (34). The two isoenzymes, A and B forms, are characterized by their sensitivity towards specific substrates and acetylinic inhibitors (9,33). MAO-A preferentially deaminates 5-HT and NA and is selectively inhibited by clorgyline, whereas MAO-B preferentially deaminates phenylethylamine (PEA) and is selectively inhibited by selegline (22). Both forms of enzyme coexist in equal proportion in the rodent brain, thus providing an ideal system for the evaluation of MAOIs. Because inhibition of MAO-A is crucial in the treatment of depression (7,33), in the present investigation, monoamine oxidase activity was determined in the rat brain synaptosomes in the presence of either dichloromethane fraction from *A. catechu* or various constituents reported to be present in it (2,17).

Behavioral experiments including forced-swim test (FST), tail-suspension test (TST), yohimbine-potentiation test (YPT), and locomotor test (LMT) related to depression were also conducted. Moclobemide (a reversible inhibitor of MAO-A) was used as a reference drug throughout the study.

Requests for reprints should be addressed to Dr. A. Dar, University of Karachi, H.E.J. Research Institute of Chemistry, Pharmacology Section, Karachi 75270, Pakistan.

METHOD

Animals

Animals were kept under standard condition with normal light cycle (12 h), with free access to food and water. The NMRI mice (23 ± 3.1 g) and Wistar rats (168 ± 5.7 g) were housed in plastic cages in groups of 10 per cage.

Drugs

Adrenaline bitartrate, arecaidine hydrochloride, arecoline hydrobromide, ascorbic acid, clorgyline, horseradish peroxidase type II, 5-hydroxytryptamine, β-phenylethylamine hydrochloride, yohimbine hydrochloride (Sigma, St. Louis, MO), sucrose (BDH, UK), l-amino acids: arginine, methionine, phenylalanine, proline, tryptophan, and tyrosine (Fluka, Switzerland), and gallic acid (Merck, Germany) were purchased from respective companies. Moclobemide was a gift from Roche, Pakistan. Selegeline hydrochloride (deprenyl) was a gift from Professor Sandler. For behavioral studies dichloromethane fraction and moclobemide were dissolved in physiological saline. In the case of the monoamine oxidase assay, all the test compounds were solubilized in distilled water. Treatments were given in a volume of 5 ml/kg and 10 ml/kg for rats and mice, respectively.

Preparation of Dichloromethane Fraction

Areca catechu nuts were purchased from a local market and authenticated at Herbarium of the Botany Department, University of Karachi. A voucher sample was submitted to the Karachi University Herbarium (KUH) with No. 67278 for reference. *Areca* nuts (10 kg) were powdered and soaked in 70% ethanol in water for a period of 6 days (13). The filtrate was evaporated and aqueous ethanol extract (266 g) was obtained. The aqueous ethanol extract was partitioned between water and hexane (1:1). Evaporation of hexane produced a gum (hexane fraction, 0.45 g) and aqueous layer that was further partitioned with dichloromethane (3 l) to afford a dichloromethane fraction (0.78 g).

Equipment

Locomotor activity of mice was monitored via an Opto-Varimex Minor (Columbus, OH). In the monoamine oxidase assay, fluorescence was measured by a spectrofluorometer (RF-1501, Shimadzu, Japan).

Experimental Procedures

Forced swim test. Male rats received an IP injection of saline (vehicle control) or dichloromethane fraction (1, 4, 7, 10, and 13 mg/kg) or moclobemide (1, 4, 7, 10, 13, 15, and 16 mg/kg) 1 h prior to the observations. Rats were placed in a glass tank (height = 45 cm, width = 17 cm) filled with water to a height of 15 cm, and temperature was maintained at 25°C. Animals were preconditioned for 15 min in a swimming tank 24 h prior to the experiment. The duration of the immobility time was recorded for a period of 5 min, and the rat was considered immobile when floating motionless (26,27). The percent reduction in the immobility time of test animals was calculated compared to the controls.

Tail-suspension test. Male mice received an IP injection of saline (vehicle control) or dichloromethane fraction (1, 2, 4, 7, 10, and 13 mg/kg) or moclobemide (1, 2, 4, 10, 13, 16, and 19 mg/kg). After 1 h of treatment mice were suspended on the edge of a table 35 cm above the ground, and the duration of the immobility time was recorded for a period of 6 min (29). Mice were considered immobile only when they hung passively and completely motionless. Percent reduction in the immobility time of test animals was calculated compared to the controls.

Yohimbine potentiation test. Male mice received saline (vehicle control) or 10, 13, 16, and 19 mg/kg dichloromethane fraction or moclobemide orally. After 30 min of treatment, all the animals received a subcutaneous injection of yohimbine, at 30 mg/kg. Mortality was observed after 24 h, and the percent mortality of the test and control animals were compared (28).

Locomotor test. A group of five mice of either sex received saline intraperitoneally 1 h before the observations. The animals were placed in activity meter, 15 min prior to the observations for acclimatization. Ten-minute locomotor counts were recorded for a period of 1 h. The mean of six such observations were noted (control counts). After 24 h, these animals received an IP injection of either dichloromethane fraction or moclobemide at 13, 16, and 19 mg/kg, and locomotor counts were recorded as described above (test counts). Test counts of each dose was compared with its respective control (13).

Preparation of synaptosomes. Rats were sacrificed by decapitation, and brain homogenate was prepared immediately in 20 vol of 0.32 M ice-cold sucrose using polytron (8000 min^{-1} for 5 s, 6×). The homogenate was centrifuged at 1000 × *g* for 10 min at 4°C, and the supernatant obtained was recentrifuged at 17,000 × *g* for 30 min. The resulting pellet was suspended in 10 vol of 0.32 M sucrose solution and homogenized using polytron as described above, aliquoted, and kept at −50°C until further use (3).

Monoamine oxidase assay. Monoamine oxidase activity was determined by the fluorometric method described previously (10,13,14) using 5-hydroxytryptamine (500 μM) and β-phenylethylamine (500 μM) as a substrate. Synaptosomes (20 μl) were preincubated with buffer or inhibitors of MAO (A and B) or test compound for 30 min at 37°C, followed by the addition of substrate (50 μl). Assay tubes were incubated for 20 min, with a final reaction volume of 200 μl. An adrenaline/peroxidase system was used to form fluorescent adrenolutine; the intensity of the fluorescence was determined using an emission wave length 405 nm with an excitation wave length 550 nm. Hydrogen peroxide (0.5–3 nm) was used as a standard throughout the experiments, and the fluorescence obtained was subtracted from the blank values to obtain the fluorescence formed per nm of H_2O_2 (125 ± 4). The control enzyme activity was calculated using this value, and expressed as nm of H_2O_2 formed h^{-1} mg^{-1} protein. The percent inhibition of enzyme activity in the presence of the test compound was determined by comparing it with the control value. Clorgyline and selegeline (irreversible MAO-A and B inhibitors) were used (0.1 μM) to confirm the presence of MAO-A and B in the rat brain synaptosomes. Protein concentration of the synaptosomes was determined by the method of Lowry et al. (18), with bovine serum albumin as a standard.

Data analysis. Data of forced-swim and tail-suspension tests were analyzed by a one-way ANOVA, followed by a least-significant difference test (19). However, Student's paired *t*-test was applied to evaluate the significance of differences in the locomotor test.

RESULTS

Both dichloromethane fraction and moclobemide significantly reduced the duration of immobility time of rats in a

TABLE 1

EFFECT OF DICHLOROMETHANE FRACTION FROM *A. CATECHU* AND MOCLOBEMIDE ON THE FORCED-SWIM TEST IN RATS

Treatment	Dose (mg/kg)	% Reduction	IC_{50} (mg/kg)
Dicholormethane	1	10 ± 2.7	
Fraction	4	34 ± 0.7	
	7	54 ± 3.1	6.8 ± 0.36
	10	76 ± 2.9	
	13	78 ± 0.4	
Moclobemide	1	24 ± 2.5	
	4	36 ± 0.5	
	7	83 ± 2.7	
	10	90 ± 0.8	5 ± 0.65
	13	92 ± 0.7	
	16	95 ± 0.5	

Number of control animals = 85.
Number of animals per dose = 4–6.
Values are mean % reduction in the immobility time ± SEM.
Immobility time of control animals = 174.4 s ± 1.8.
All the values showed significant percent reduction ($p < 0.05$) in the mobility time compared to the control.
IC_{50} = The dose that produced 50% reduction in the immobility time.

dose-dependent manner, in contrast to the control animals. Dichloromethane fraction showed a maximum reduction of 76% at 10 mg/kg, and its magnitude was about 20% less than that caused by moclobemide. The corresponding IC_{50} values for dichloromethane fraction and moclobemide were 6.8 ± 0.36 and 5 ± 0.65 mg/kg (Table 1). In both cases, the reduction in immobility was accompanied at the higher doses by the signs of excitation, which was reflected as increased struggling behavior for floating upright in the water. In another pharmacological model of depression, dichloromethane fraction showed a weak dose dependent decline in the immobility time of mice with a maximum reduction of only 32% (4 mg/kg), reaching a plateau, followed by a reversal of this particular behavior at higher doses (Table 2). On the other hand, mocolobemide also showed similar pattern of reduction and reversal of behavior, but the extent of reduction in immobility time was 60% greater than that of dichloromethane fraction. The respective IC_{30} values of 2.4 ± 0.6 and 1.9 ± 0.13 mg/kg were obtained for dichloromethane fraction and moclobemide.

TABLE 2

EFFECT OF DICHLOROMETHANE FRACTION FROM *A. CATECHU* AND MOCLOBEMIDE ON THE TAIL-SUSPENSION TEST IN MICE

Treatment	Dose (mg/kg)	% Reduction	IC_{30} (mg/kg)
Dicholormethane	1	15 ± 0.6	
Fraction	2	25 ± 3.2	
	4	32 ± 2.8	
	7	30 ± 2.2	2.4 ± 0.6
	10	25 ± 2.2	
	13	17 ± 0.2	
Moclobemide	1	20 ± 0.7	
	2	26 ± 4	
	4	49 ± 5.1	
	10	53 ± 3.4	1.9 ± 0.13
	13	93 ± 2.2	
	16	41 ± 0.7	
	19	24 ± 2.8	

Number of control animals = 124 and number of animals per dose = 4–10.
Values are mean % reduction in the immobility time ± SEM.
Immobility time of control animals = 119.7 s ± 1.3.
All the values showed significant percent reduction ($p < 0.05$) in the mobility time compared to the control.
IC_{30} = The dose that produced 30% reduction in the immobility time.

TABLE 3

EFFECT OF DICHLOROMETHANE FRACTION FROM *A. CATECHU* AND MOCLOBEMIDE ON THE YOHIMBINE-POTENTIATION TEST IN MICE

	Percent Mortality	
Dose (mg/kg)	Dichloromethane Fraction	Moclobemide
10	10	10
13	30	30
16	40	50
19	50	70

Number of control animals = 60.
Number of animals per dose = 10.
Percent mortality in control animals = 10.

Subcutaneous treatment of yohimbine (30 mg/kg) caused about 10% mortality in the control mice. Pretreatment of animals either with dichloromethane fraction or moclobemide before yohimbine administration caused a dose-dependent increase in the mortality, reaching a value of 50 and 70%, respectively, at 19 mg/kg (Table 3).

Table 4 shows that the locomotor activity of animals did not change significantly after the administration of 19 mg/kg of dichloromethane fraction, whereas at the same dose moclobemide showed a significant reduction (53%) in the motor activity.

TABLE 4

EFFECT OF DICHOLOROMETHANE FRACTION FROM *A. CATECHU* AND MOCLOBEMIDE ON THE LOCOMOTOR TEST IN MICE

Treatment	Dose (mg/kg)	Locomotor Counts	
		Control	Test
Dichloromethane	13	4528.3 ± 719.2	4746.3 ± 296.9
Fraction	16	7026.3 ± 914.2	7289.7 ± 1017.3
	19	6404.7 ± 790.2	580.7 ± 542.2
Moclobemide	13	7073.5 ± 235.8	8916.8 ± 1081.2
	16	5550.5 ± 623.5	4175.7 ± 515
	19	5238.3 ± 496.3	2461.5 ± 360*

Number of animals per dose = 5.
Values are mean locomotor counts/10 min ± SEM.
Asterisks indicate significant reduction ($p < 0.005$*) in the locomotor counts, and all the other values showed nonsignificant differences compared to their respective control.

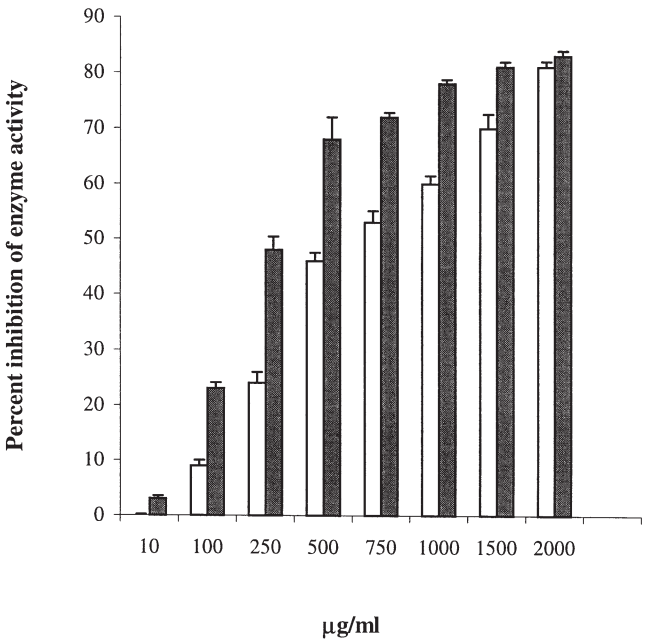

FIG. 1. Effect of various concentrations of dichloromethane fraction (□) or moclobemide (■) on MAO-A activity. Samples of synaptosomes (26.4 ± 1.3 mg/ml protein) were preincubated either with dichloromethane fraction or moclobemide for 20 min at 37°C before the addition of 5-hydroxytryptamine (500 μM). Inhibition of enzyme activity is expressed as a percentage of activity remaining of the control sample preincubated without the inhibitor. Each point is the mean (±SEM) of four to six determinations, each in duplicate or triplicate.

TABLE 5

EFFECT OF DIFFERENT CONSTITUENTS OF *A. CATECHU* ON MONOAMINE OXIDASE-A

Test compound	Concentration (μg/ml)	% Inhibition of Enzyme Activity
Arecaidine	150	0
Arecoline	2000	0
Arginine	150	0
Catechine	150	0
Gallic acid	100	0
Methionine	150	0
Phenylalanine	150	0
Proline	150	0
Tryptophan	150	0
Tyrosine	150	0
Arecaidine + Arecoline	150	0
Mixture-1	150	0
Mixture-2	150	0
Mixture-3	150	0

Samples of synaptosomes (26.4 ± 1.3 mg/ml protein) were preincubated with various test compounds for 20 min at 37°C before the addition of 5-hydroxytryptamine (500 μM). Inhibition of enzyme activity is expressed as a percentage of activity remained of control sample preincubated without test compound. Each point is the mean (± SEM) of two to three determinations, each in duplicate or triplicate.

Mixture-1 = Arecaidine + arecoline + arginine + catechine + methionine + phenylalanine + proline + tryptophan + tyrosine.

Mixture-2 = Arecoline + arginine + catechine + methionine + phenylalanine + proline + tryptophan + tyrosine.

Mixture-3 = Arecaidine + arginine + catechine + methionine + phenylalanine + proline + tryptophan + tyrosine.

The effect of dichloromethane fraction and moclobemide on enzyme activity of the rat brain MAO-A using 5-HT as a substrate are presented in Fig. 1. The enzyme activity was measured in the untreated samples (control) and in the presence of dichloromethane fraction or moclobemide. It was expressed as percent inhibition of enzyme activity of control samples, which was 20.2 ± 1 nm of H_2O_2 h^{-1} mg^{-1} protein. It is clear that dichloromethane and moclobemide, when tested over a dose range of 10 to 2000 μg/ml, showed a concentration-dependent inhibition of MAO-A, reaching to a value of 80%. No such inhibition was seen with various costituents of *A. catechu* such as arecaidine, arecoline, arginine, catechin, gallic acid, methionine, phenylalanine, proline, tryptophan, and tyrosine (Table 5). The table also shows that the enzyme activity remained unchanged in the presence of various mixtures of the aforementioned constituents of the *Areca* nut.

To examine the effects of selegeline on MAO-B, rat brain synaptosomes were preincubated with 1 μM selegeline for 30 min using PEA as a substrate. The enzyme activity was completely inhibited; however, dichloromethane fraction at 1000 μg/ml did not affect the MAO-B activity (data not shown).

DISCUSSION

Our earlier studies showed that aqueous ethanol extract, hexane, ethyl acetate, and aqueous fractions from *A. catechu* possess antidepressant properties (11–13). Current investigation has been extended to see whether this property has been improved or not in the dichloromethane fraction from the nut.

It has previously been shown that behavioral studies play an important role for the evaluation of antidepressant drugs and reduction in immobility time of animals reflects their antidepressant property (6,33). Both dichloromethane fraction and moclobemide (MAO-A inhibitor) caused a significant reduction in the immobility time, using forced-swim and tail-suspension tests to support the above statement. These results are in accordance with our earlier findings with nut extract and its respective fractions from *A. catechu* (11–13), and also with various clinically active antidepressants such as nialamide, toloxatone, and amitriptyline (5,24,25,30,32).

Potentiation of yohimbine lethality in mice is also considered as a classical screen (6), and is widely used for the assessment of antidepressant drugs (28). The fraction and moclobemide appeared to potentiate yohimbine-induced lethality, indicating that it may have occurred due to an increase in monoamines, possibly noradrenaline in the brain and peripheral tissues (8,33).

Dichloromethane fraction and other antidepressants such as SKF 38393 and A 68930 had no effect on motor activity (1), whereas moclobemide produced a significant decline in the motor performance at 19 mg/kg, that was similar to desipramine, iproniazid, and mianserin (25). Considering the variable effects of antidepressants on the locomotor activity, dichloromethane fraction appears to be more selective towards its antidepressant action. This property of dichloromethane fraction is unlikely to be related to the stimulant actions of the *Areca* nut, as reported earlier (21).

It has been postulated that the functional deficiencies of monoamines (5-HT and NA) at the neuronal level is mainly responsible for depression (8), and monoamine oxidase inhib-

itors exert their therapeutic action by increasing the availability of monoamines at the neuronal level (20). It was suggested earlier that alkaloids and some other compounds present in the *Areca* nut might be responsible for MAO-inhibiting property (13). Therefore, few constituents of *A. catechu*, i.e., arecaidine, arecoline, arginine, catechin, gallic acid, methionine, phenylalanine, proline, tryptophan, and tyrosine (2,17), were evaluated for their MAO-inhibiting activity using rat brain synaptosomes as an enzyme source. The fact that the enzyme activity remained unchanged in the presence of the above-mentioned compounds rules out their possible interaction with this enzyme. However, dichloromethane fraction and moclobemide showed a clear-cut dose-dependent inhibition of monoamine oxidase using 5-HT as a substrate. Although the IC_{50} value for dichloromethane fraction (665 ± 65.1 μg/ml) was lowest compared to those obtained for extract and other fractions from *A. catechu* (13), it was 50% greater than that obtained for moclobemide (280 ± 13.7 μg/ml). Thus, the dichloromethane fraction from the *Areca* nut appears to be more potent than its extract and other fractions, but less potent than moclobemide, thus indicating that dichloromethane fraction containing many compounds required bioassay-directed fractionation to obtain the active moiety. It is worth noting that an amine such as PEA has been shown to be deaminated by MAO-B (22) and the lack of effect of dichloromethane fraction on PEA deamination, despite the strong reduction in 5-HT deamination in rat brain synaptosomes, suggests that dichloromethane is a selective inhibitor of MAO-A.

In the light of the present findings, it may be concluded that dichloromethane fraction from *A. catechu* exhibit an antidepressant property via monoamine oxidase type A inhibition. Further studies are required to identify the active principle responsible for this action.

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