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Chronic Contamination in Rats by Reduced Risk Pesticides: Cases of Spirotetramat and *Citrullus Colocynthis* (*Cucurbitaceae*) Extracts

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ABSTRACT

The use of pesticides becomes an indispensable technique for most agricultural practices, whatever the level of country development. At present, the pesticides used are often less toxic and more specific and are based on insect physiology. Recent studies indicate that pesticide intoxication induces oxidative stress.
This work aims to study the neuro-behavioral consequences of the administration of two insecticides currently widely used in agriculture: spirotetramat (inhibitor of lipid synthesis in insects) and ethanol extract of Citrullus colocynthis (endemic plant of the Algerian Sahara). The different behavioral tests (raised cross labyrinth, open fields and forced swimming) show that both pesticides had a significant impact on rodent's anxiety degree. These products significantly influence the biochemical parameters (glycemia, cholesterol, triglycerides, urea, creatinine), the ACTH hormone and acetylcholine esterase (AChoE).

Keywords: *insecticides, spirotetramat, Citrullus colocynthis, non-target organism, Wistar rat.*

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INTRODUCTION

Following last reports, annual global pesticide expenditures represent more than 25 billion Euros for a total amount of about 2.5 million tons of pesticides (FAO, 2012; Fenner et al., 2013). Moreover, 90% of these products are lost in the air, at the application time or due to the runoff phenomenon, affecting both the treatment costs and ecosystems (Ghormade et al., 2011). In order to control the pest populations and to limit their proliferation, man is making considerable efforts and looking for new methods of physical, chemical and biological control (Kim et al., 1995, Lyon, 1997). Insecticides are substances that allow the arthropods elimination. They can be used against larvae and/or eggs (larvicides) or against adults. They must be harmful to insect pests but also relatively harmless to non-target organisms (Louat, 2013).
Chemical control is a method of using chemical plant protection products to control pests. She has increased risks of developing the cancer kinds, a drop in male fertility, hormonal disturbances, immune problems, congenital malformations ... etc. Among the most serious effects, some can even be transmitted from one generation to another (Veillerette, 2005). The risk assessment of chemical control can adequately reflect the hazards of these products to humans and environment.
As a result of the harmful consequences of chemical control on environment and on humans, which is reflected in carcinogenic phenomena (El Sayed & Donelson, 1997, Ishaaya & Horowitz, 1998), biological control has just been integrated in order to fight against pests. Natural pesticides, resulting from biotechnology development, are defined broadly as coming from living organisms: animals, plants, bacteria or certain minerals (Sporleder & Lacey, 2013). According to environmental protection agency (EPA), more than 192 active organic pesticides have been registered (Cantrell et al., 2012). Biological control takes several forms, but, now, is natural substances of plant origin that attracts researcher's attention (Boutaleb-Joutei, 2010). Spontaneous plants in arid area are considered the plant genetic resources of agronomic, economic, ecological and strategic interests (UNESCO, 1960).
Pesticide toxicity studies are often limited to various forms of direct and acute toxicity in target organisms and non - target organisms. Indeed, some harmful neurotoxic pesticides effects are related to another form of toxicity on developing organism. It's called developmental neurotoxicity and it's therefore linked to an "environmental exposure" in which some pesticides have a key role. Several studies have shown that gestational or neonatal exposure to pesticides causes neurobehavioral alterations in offspring in different developmental phases such as those of Lassiter et al (1998), Levin et al (2002), Laura et al (2004) and Chanda & Pope (1996).
This study has objective to determine the efficacy and neuro-behavioral consequences of administering two pesticides widely used in agriculture. The first one is a reduced-risk chemical, spirotetramat, which is an inhibitor of lipid synthesis in insects and the second product is the ethanolic extract of Citrullus colocynthis (Cucurbitaceae) (common name: hantel), a plant endemic to the Algerian Sahara.

MATERIALS & METHODS

2.1. Animal:

For all experiments, we used adult rats "Rattus rattus" - Wistar strain from the Pasteur Institute (Algiers, Algeria). Rats were raised in sawdust-lined plastic cages with a steel lid and baby bottles filled with water. Rat's food is made in the sticks form consisting of corn, barley, milk and vitamin supplement. These animals were acclimatized to laboratory conditions (temperature 25 ± 2 ° C and humidity 70 - 80% and photoperiod 12:12h).

2.2. Spirotetramat:

Spirotetramat is a spirocyclic tetramic acid derivative, it's the only foliar insecticide and exclusively developed by Bayer CropScience. The product has a novel and unique action mode, classified as an inhibitor of acetyl CoA carboxylase or an inhibitor of insect's lipid biosynthesis. Spirotetramat is an oral intoxicant and is active mainly on immature insects (Bell, 2013).

2.3. Citrullus colocynthis (Cucurbitaceae):

It's a spontaneous plant in arid area of Africa and Asia, It's very common in Sahara. Used in traditional medicine to treat excess sugar, which explains the intoxications frequency in the Maghreb (Hammiche et al., 2013). The toxicity is due to cucurbitacins and their glycosides present throughout the plant, particularly in fruit and seeds (Darwish-Sayed et al., 1974, Seger et al., 2005). The plant used in this study was collected in south central Algeria (Laghouat) (33 ° 48'24 "north latitude, 2 ° 52'56" east longitude) and we used the seeds for extraction with ethanol.

2.4. Pesticide treatments:

To studied indirect effects of Spirotetramat, twenty rats were divided into two groups: a control group (10 individuals: 5 males and 5 females) and a treated group (10 individuals: 5 males and 5 females). These animals have 10% of spirotetramat (15µg/ml) during 7 days (every other day).

For C. colocynthis, thirty rats were separated into two groups, a control group (10 individuals: 5 males and 5 females) and a treated group (20 individuals: 10 males and 10 females). These animals have 2ml of C. colocynthis (20µg/ml) for 7 days (every other day) (treatment by gavages).

2.5. Rat's Behaviors in different anxiety-provoking situations:

We tested the different rats groups using experimental devices recognized by the scientific society whose most used are the plus maze (EPM) (Rodgers & Dalvi, 1997; VanGaalen & Steckler, 2000; Karl et al., 2003; Elizalde et al., 2008), the open field (OP) (Crawley, 1999; Palanza, 2001; Karl et al., 2003; Prut & Belzung, 2003; Elizalde et al., 2008) and the forced swimming test or Porsolt test (FST) (Porsolt et al., 1977; Karl et al., 2003; Elizalde et al., 2008).

2.6. Effect on certain biochemical parameters:

Blood is collected from control adults, Spirotetramat's adults and C. colocynthis's adults. The separated plasma is frozen and stored immediately at (-20°C). ACTH plasmas were measured by radioimmunoassay (Raff et al., 2004). We also performed a blood glucose, cholesterol, triglyceride, urea, creatinine and AchoE (Acetyl Choline Esterase) assay.

2.7. Data Analysis:

The various study data were analyzed by descriptive and comparative methods (variances analysis) on XLStat 2009 software. The multivariate analysis (MANOVA) with SPSS Statistics 22.0 enabled us to test treatment and sex effects on the rat behaviors tested and biochemical parameters.

RESULTS & DISCUSSION:

3.1. Behaviors of rats in different anxiety-provoking situations:

3.1.1. Plus maze test:

This test is used to evaluate anxiety about vacuum (Kousteff, 2011). The rat is anxious and naturally takes refuge in the closed arms which offer him greater security and will hesitate to explore with open arms (Rodgers & Dalvi, 1997; vanGaalen & Steckler, 2000; Karl et al., 2003; Elizalde et al., 2007; Kousteff, 2011). Our results show that there are no significant differences between times spent in closed arms and/or open arms in control and treated rats for both sexes (Table 1). A short time spent in open arms is considered to be an anxiety index (Onaivi et al., 1990; Lister, 1987; Pellow et al., 1985); this index is 11 to 22 seconds for males and 15 to 25 seconds for females. However we have recorded that entry's number in closed arms and/or the open arms is a little higher in control's rats; the treated rats are less active and make a single choice when put in the device plus maze (Tab.1). The decrease in open-arm exploration is typically interpreted as an anxiety increase (Elliott et al., 2004). The variances comparison shows that spirotetramat and C. colocynthis extract are reduced risk products, unlike the "Diazinon" insecticide, for example. According to Tayaa (2014), Diazinon acts significantly on females' rat behavior in the plus maze.

Table 1: Pesticides effect on rat's behavior (in plus maze)

N=10	Time spent in closed arms	Time spent in open arms	Number of closed arms	Number of open arms
♂C	243.20±12.46	18.00±24.22	5.40±2.70	0.80±0.84
♂S	269.20±6.30	11.00±4.00	3.80±0.58	0.80±0.37
♂Cc	255.50±24.05	22.58±26.04	2.93±1.29	1.21±1.06
F_{obs}	**0.697**	**1.729**	**1.200**	**0.168**
p	**0.512**	**0.207**	**0.326**	**0.847**
♀C	257.00±24.40	25.80±23.00	2.80±1.48	2.00±1.58
♀S	242.80±11.91	15.20±5.11	4.00±0.84	1.40±0.51
♀Cc	275.40±26.71	14.80±21.85	1.70±0.95	0.70±1.06
F_{obs}	**0.347**	**1.994**	**0.600**	**0.540**
p	**0.711**	**0.167**	**0.560**	**0.592**

[C: Control; S: spirotetramat; Cc: C. colocynthis] [* significant*; ** highly significant; *** very highly significant

3.1.2. Open Field Test:

This test evaluates an animal's response to a new and spacious environment (Kousteff, 2011). It creates a conflict situation between the animal's natural tendency to explore this new environment and its aversion to open spaces (Kousteff, 2011). As a result, normal rats tend to spend more time in corners and periphery of open field (the open field's center is considered the most anxiety-provoking area) (Crawley, 1999; Palanza, 2001; Karl et al., 2003; Prut & Belzung, 2003; Elizalde et al., 2008). The Table 2 results show that control and treated rats prefer to remain in the open field periphery. Entries number in the open field center is 10 to 20 times smaller (Table 2). It also appears from this table that the defecation number, recovery and immobility decreases when we treat rats with C. colocynthis; these three parameters are relatively higher in females than in males (Table 2). This test involves measuring locomotor activity (Tayaa, 2014). Spirotetramet and C. colocynthis do not significantly influence this activity. Some products cause acute locomotor hypoactivity such as organophosphorus pesticides (Tayaa, 2014); GABAergique system disruptions involve anxiety disorders (Möhler, 2006;

Domschke & Zwanzger 2008). There is a lot of scientific research that has highlighted the system role in anxiety disorders (Tayaa, 2014).

Table 2: Pesticides effect on rat's behavior (in open field)

N=10	Number of entrances in the clear areas of the center	Number of entrances in the light areas of the periphery	Number of entrances in the dark areas of the center	Number of entrances in dark areas of the periphery	Number of defecations	Number of adjustments	Number of immobility
♂C	3.40±2.07	54.00±10.49	6.60±2.79	65.00±14.21	2.00±1.00	101.40±24.19	21.40±9.94
♂S	3.40±1.30	52.00±3.92	7.60±1.54	63.80±3.007	2.00±0.84	129.20±19.93	35.60±2.14
♂Cc	2.60±2.07	42.60±36.47	3.40±2.41	43.60±38.05	1.80±1.32	36.10±29.42	30.60±20.61
F_{obs}	**0.328**	**8.401**	**0.980**	**7.187**	**2.165**	**0.518**	**3.563**
p	**0.724**	**0.003****	**0.396**	**0.005****	**0.145**	**0.605**	**0.051**
♀C	6.75±3.78	125.80±20.14	9.40±2.70	54.90±35.07	2.80±2.39	78.60±17.50	76.00±21.31
♀S	5.20±2.2	43.80±3.50	11.20±3.99	52.40±5.77	3.60±1.21	122.00±22.65	32.20±3.15
♀Cc	2.11±1.90	51.60±29.46	2.80±2.39	2.80±2.39	2.60±2.55	34.4 ± 16.73	23.90±15.28
F_{obs}	**3.318**	**2.887**	**14.817**	**1.744**	**0.195**	**4.458**	**1.777**
p	**0.061**	**0.083**	**0.000*****	**0.205**	**0.825**	**0.028***	**0.199**

[C: Control; S: spirotetramat; Cc: C. colocynthis] [* significant; ** highly significant; *** very highly significant]

3.1.2. Forced swim test:

This test is to put the rat in a dangerous situation where he must struggle to survive, but without flees' possibility. The forced swimming test or the antidepressant efficacy test represents an aversive and stressful situation where the rat can't escape and produces immobility and despair behavior (Porsolt et al., 1978; Kirby & Lucki, 1997). In animals, immobility is interpreted as a lack of will to survive and considered as a depression sign (Porsolt et al., 1977, Petit-Demouliere et al., 2005). The results show that both pesticides have a significant effect on swimming time, which is 2 to 5 times lower for both sexes (Table 3). We recorded that the escalation time and/or immobility time are greater in these treated individuals (Table 3). This result confirms the increase in depression signs caused by pesticides used in this study.

Table 3: Pesticides effect on depressive state

	Swimming time	Climbing time	Time of immobility
♂C	**111.20±28.68**	**79.00±22.29**	**122.40± 29.01**
♂S	**66.40±23.65**	**158.80±28.37**	**74.60±13.17**
♂Cc	**35.10±24.24**	**46.00±43.90**	**218.90±51.96**
F_{obs}	**1.074**	**1.966**	**1.718**
p	**0.364**	**0.171**	**0.209**
♀C	**147.20±50.69**	**40.80±29.69**	**103.00±34.48**
♀S	**43.00±16.18**	**174.00±29.70**	**63.20±17.67**
♀Cc	**28.40± 19.29**	**105.90±37.57**	**165.70±41.82**
F_{obs}	**6.618**	**1.242**	**0.200**
p	**0.007****	**0.314**	**0.821**

[C: Control; S: spirotetramat; Cc: C. colocynthis] [* significant; ** highly significant; *** very highly significant]

3.2. Pesticides effect on biochemical parameters:

We recorded a significant decrease in ACTH hormone level in rats treated with spirotetramat (Tab.4), which is a chemical insecticide marketed as a reduced risk product. The C. colocynthis extract has two different effects; in males we recorded a significant increase in plasma hormone which reached 536.80±137.99 pg/ml. This quantity is 5 times greater than control males (Table 4). In C. colocynthis females, we recorded a significant decrease in ACTH hormone (4 times less than the control) (Table 4). For biochemical parameters, we did not record any significant effects of the two products (Table 4).

Table 4 Pesticides effect on various biochemical parameters, ACTH hormone and AChoE

	Glycemia (g/l)	Cholesterol (g/l)	Triglyceride (g/l)	Urea (g/l)	Creatinine (mg/l)	ACTH (pg/ml)	AChoE (nmol/min/mg Protein)
♂C	**1.02±0.16**	**0.44±0.04**	**0.75±0.069**	**0.51±0.071**	**3.85±0.29**	**89.19±31.40**	**0.16±0.04**
♂S	**0.95±0.06**	**0.39±0.04**	**0.61±0.01**	**0.69±0.02**	**2.95±0.11**	**14.47±8.08**	**0.13±0.05**
♂Cc	**1.17±0.23**	**0.61±0.15**	**0.49±0.18**	**0.60±0.12**	**5.06±0.34**	**536.80±137.99**	**0.07±0.03**
F_{obs}	**2.830**	**0.548**	**9.467**	**2.045**	**2.425**	**4.594**	**0.588**
p	**0.098**	**0.591**	**0.003****	**0.172**	**0.103**	**0.032***	**0.570**
♀C	**0.82±0.045**	**0.43±0.048**	**1.12±0.25**	**0.35±0.024**	**3.41±0.56**	**100.08±29.93**	**0.19±0.03**
♀S	**0.98±0.08**	**0.38±0.04**	**0.62±0.12**	**0.49±0.01**	**4.99±0.31**	**19.24±16.72**	**0.13±0.04**
♀Cc	**1.10±0.15**	**0.56±0.08**	**0.55±0.30**	**0.62±0.15**	**6.72±0.47**	**22.69±30.58**	**0.09±0.02**
F_{obs}	**0.493**	**1.004**	**2.581**	**1.668**	**1.250**	**3.039**	**0.754**
p	**0.622**	**0.394**	**0.116**	**0.229**	**0.321**	**0.05***	**0.491**

[C: Control; S: spirotetramat; Cc: C. colocynthis] [* significant; ** highly significant; *** very highly significant]

We studied the effect of sex and different treatments (spirotetramat and C. colocynthis extract) on Wistar rat's anxiety state. MANOVA statistical analysis using the Lambda Wilk's test shows a highly significant effect of pesticides treatment ($F_{19.6}$= 18.39, p: 0.000) and sex-treatment association on rats anxiety behavior ($F_{38.12}$= 3.81, p: 0.008) (Table 5). It seems the sex factor does not significantly affect the rats anxious behavior (p: 0.191, NS) (Table 5).

Table 5: Treatment Effect on the different parameters studied (Multivariate test: Lambda Wilk's test)

Effect	Value	F	Hypothesis df	Error df	Sig
Sex	0.134	2.05	19.00	6.00	**0.191**
Treatment	0.000	18.39	38.00	12.00	**0.000*** **
Sex * Treatment	0.006	3.81	38.00	12.00	**0.008** **

[* significant; ** highly significant; *** very highly significant]

The treatment effects mainly reside in the entries number at plus maze closed arms (F= 3.852, p: 0.04) (Table 6), the entries number into the light/dark areas open field, the entries number in open field periphery's (F=9.098 ; p : 0.000 / F= 7.896 ; p : 0.05) (Table 6), the recovery and immobility in open field (F= 15.054, p: 0.000 / F= 3.397, p: 0.000) (Table 6) and swimming time (F= 14.915, p: 0.000) (Table 6). With the exception of blood glucose, we recorded highly significant treatment effects on ACTH hormone level (F= 48.694, p: 0.000) (Table 6), cholesterol (F= 9.613, p: 0.000) (Table 6), triglycerides (F= 5.325, p: 0.01) (Table 6), urea (F= 8.163, p: 0.000) (Table 6), creatinine (F= 26.623 p: 0.000) (Table 6) and AChoE (F= 16.989, p: 0.000) (Table 6). The sex factor influences mainly entries number into the plus maze closed arms and the entries number into the light/dark areas of open field periphery and the swimming time. ACTH and urea also depend on this sex factor (Table 6). The combination of the two factors (sex - treatment) confirms these results (Table 6).

In mammals, spatial memory is regulated by cortex and hippocampus association as well as by the temporal lobe system, which is the link between these two structures (Canto et al., 2008). If many neurotransmitter systems (glutamate, acetylcholine, GABA), are involved in memory processes (Ogren et al., 2008). It is therefore logical to think that AChE inhibition must participate in the mechanisms that cause these persistent changes in rats' spatial memory. In addition, results from Tayaa (2014) indicated that prolonged acetylcholinesterase inhibition caused by repeated exposure to organophosphorus compounds alters the spatial memory functions.

Table 6: Effects of the treatment and / or sex factor on the studied parameters (inter-subject effects)

Source	Sex					Treatment					Sex * Treatment				
	SSIII	ddl	MS	F	Sig	SSIII	ddl	MS	F	Sig	SSIII	ddl	MS	F	Sig
Recovery-PM	45.63	1	45.63	1.64	0.21	142.2	2	71.1	2.548	0.1	358.867	2	179.433	6.43	0.01**
Closed Arms-PM	13.67	1	13.67	4.97	0.04*	21.20	2	10.602	3.852	0.04*	10.138	2	5.069	1.842	0.18
Open arms-PM	2.03	1	2.03	2.01	0.17	1.182	2	0.591	0.584	0.57	2.607	2	1.303	1.289	0.29
Center-OF Clear Zone	9.63	1	9.63	1.15	0.29	24.27	2	12.13	1.45	0.25	46.667	2	23.333	2.789	0.08
Clear zone periphery-OF	4588.03	1	4588.03	8.59	0.01**	9717.07	2	4858.53	9.098	0.000***	8749.067	2	4374.533	8.191	0.000***
Dark zone center-OF	28.03	1	28.03	1.39	0.25	176.47	2	88.23	4.379	0.02*	24.867	2	12.433	0.617	0.55
Dark zone periphery-OF	4440.83	1	4440.83	6.5	0.02*	10764.87	2	5382.43	7.896	0.000***	8220.467	2	4110.233	6.03	0.01**
Defecation-OF	5.63	1	5.63	1.41	0.25	1.4	2	0.7	0.175	0.84	2.467	2	1.233	0.308	0.74
Recovery-OF	1116.30	1	1116.30	0.95	0.34	35288.87	2	17644.43	15.054	0.000***	421.8	2	210.9	0.18	0.84
Immobility-OF	1672.53	1	1672.53	7.52	0.01**	1511.27	2	755.63	3.397	0.05*	5911.667	2	2955.833	13.287	0.000***
Swimming Time-FST	43.20	1	43.20	0.03	0.86	53089.4	2	26544.7	18.384	0.000***	4638.6	2	2319.3	1.606	0.22
Immobility-FST	25346.13	1	25346.13	15.46	0.000***	48913.8	2	24456.9	14.91	0.000***	27472.07	2	13736.03	8.377	0.000***
ACTH	207075.24	1	207075.24	55.30	0.000***	364695.36	2	182347.68	48.694	0.000***	454205.683	2	227102.842	60.646	0.000***
glycemia	0.05	1	0.05	1.00	0.33	0.258	2	0.13	2.792	0.08	0.065	2	0.033	0.707	0.5
Cholesterol	0.004	1	0.004	0.35	0.56	0.209	2	0.10	9.613	0.000***	0.004	2	0.002	0.175	0.84
triglyceride	0.17	1	0.17	1.88	0.18	0.962	2	0.48	5.325	0.01**	0.197	2	0.099	1.093	0.35
Urea	0.10	1	0.10	8.83	0.01**	0.189	2	0.09	8.163	0.000***	0.072	2	0.036	3.106	0.06
creatinine	1.30	1	1.30	2.69	0.11	25.688	2	12.84	26.623	0.000***	6.064	2	3.032	6.284	0.01
AchoE	0.003	1	0.003	2.02	0.17	0.046	2	0.02	16.989	0.000***	0.003	2	0.001	0.946	0.4

(PM: Plus Maze; OP: Open Field; FST: Forced swimming test) [* significant; ** highly significant; *** very highly significant

CONCLUSION:

In this study we were able to show the both pesticide products effect on a non - target organism. The two pesticides studied caused behavioral disturbances in the Wistar rat as a result of ACTH hormone levels disturbances as well as spatial memory disturbances due to the products effect on the AchoE rate. It seems that the extract of Saharan plant C. colocynthis presents less risk compared to the lipid synthesis inhibitor (spirotetramat) and for this we recommend the use of bioactive molecules from plants in the biological fight against pests.

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