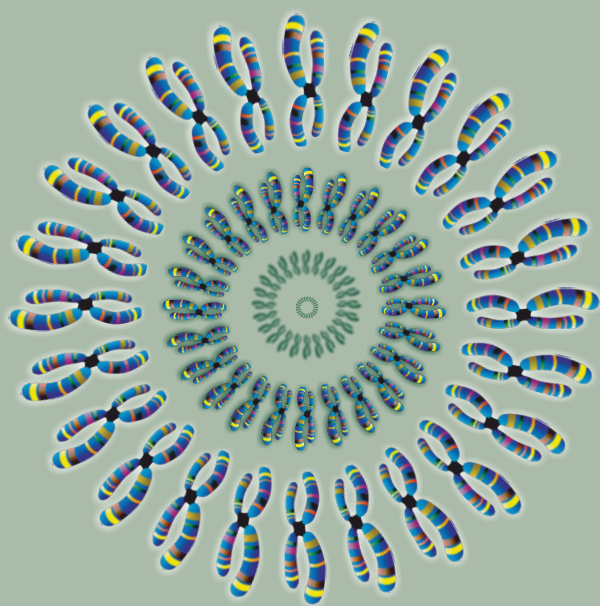
ALEXANDRU IOAN CUZA UNIVERSITY OF IASI

JOURNAL OF EXPERIMENTAL AND MOLECULAR BIOLOGY

Volume 24 Number 1 (2023)

ISSN 2601 – 6974



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„ALEXANDRU IOAN CUZA” UNIVERSITY OF IAȘI

JOURNAL OF EXPERIMENTAL AND MOLECULAR BIOLOGY

TOME XXIV, Number 1 **2023**

Editura Universității „ALEXANDRU IOAN CUZA” din Iași

EDITORIAL OFFICE

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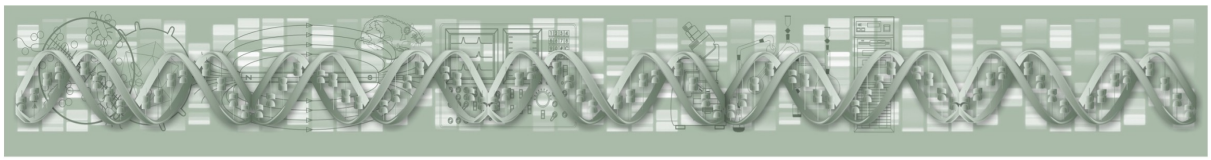

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IMPACT OF BOTANICAL SPICES IN CONTROL OF BAMBARA NUT PESTS *CALLOSOBRUCHUS SUBINNOTATUS* (COLEOPTERA, BRUCHIDAE)

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Abstract

The present study evaluated the effect of extracts of three spices in control of *Callosobruchus subinnotatus* (Pic). The experimental design used was completely randomized design (CRD). Ginger, scent leaf and curry leaf were obtained and screened for phytochemical and proximate composition using standard laboratory techniques. The Bambara nut was subjected to three treatments (T_o, T_1, T_2 and T_3) which were replicated 4 times. T_o was used as control while the rest were treated with the spices. Results from the study showed that the highest mortality rate was recorded on 100g of Ginger extract having percentage mortality of 93.33% (T_3). It was followed by scent leaf with a mortality rate of 43.33% (T_1). The result also showed that Ginger powder extract gave the lower mean number of holes (41) and with lower weight loss of 4.9% of nuts amongst the extracts used. Bambara nuts that were not treated with plant spice extracts (T_o) gave the highest mean number of holes or punctures (211) and the highest weight loss 25.5% of nuts. Conclusion of this study is that the plant extracts were effective at controlling *Callosobruchus subinnotatus*, though at varying degrees. These biopesticides are part of humans and animals food, often are used as ethno-medicines, and are more environmentally friendly being biodegradable, so all these things recommend to be used in the detriment of chemical pesticides.

Keywords: Botanical spices, Bio-insecticides, *Callosobruchus subinnotatus*, Biodegradable

Introduction

World's population stands at about 8 billion (FAO 2015) and it is predicted to increase with 2.2% per year to around 11.5 billion bin2100, with 87% living in the developing countries of Africa, Asia and Latin America (Penning de Vries 2001). The high population growth rate, particularly in the developing countries, and the changing diets will lead to a much higher quantity of food demand by 2020 (Penning de Vries 2001). Enhancing food availability in sub-Saharan Africa could be realized not only by increasing agricultural productivity through the use of sustainable good agricultural practices, but also by reducing pre- and post-harvest crop losses (Tscharntke et al. 2012). In the dry African Saharian countries agricultural production is seasonal while demands for agricultural commodities are more evenly spread throughout the year (Mikolo et al. 2007). In this circumstance, grains need to be stored from one harvest to the next one in order to maintain its constant supply all year round and to preserve its quality until required for use (Nukenine 2010). The reduction of postharvest grain losses, especially those caused by insects, microorganisms, rodents, and birds, can increase available food supplies, particularly in less developed countries where the losses are largest and the need is greatest. Amongst these living organisms, insects are responsible for the greatest storage losses in cereals and pulses (Kosini and Nukenine 2017). Bambara nut [*Vigna subterranean* (L.) Verdc] is an indigenous African legume crop which is cultivated

Received 1 August 2022; Accepted 26 January 2023; Published Online 31 January 2023; Published 3 April 2023

throughout sub-Saharan Africa, especially in the drier driest? parts of the continent (Mkandawire 2007).

Bambara nut is produced mainly as a subsistence crop, usually by small-scale female farmers. The crop is grown primarily for its seeds which are eaten fresh when semi-ripe and as a pulse when dry and mature, or ground into flour. Bambara nut is a major source of vegetable protein in sub-Saharan Africa where it constitutes an important part of the local diet, culture and economy. The seed is regarded as a completely balanced food (Rowland 1993, Anchirina et al. 2001). Bambara nut seeds, haulm and dry leaves have been used to feed livestock and poultry (Ancchirina et al. 2001). It is a rich source of minerals, energy and protein, with as much as 25.2% protein, 65% carbohydrates and 6% lipid, on a dry weight basis. Its tolerance to drought and poor soils which makes it ideally suited to production in marginal areas where low-input arable agriculture is the norm (Amarteifio et al. 2006).

Traditionally, the grain weevils, *Sitophilus sp.* (Coleoptera: Curculionidae), the Angoumois grain moth, *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) and three genera of bruchids, *Acanthoscelides, Zabrotes,* and *Callosobruchus spp.* are the most important pests of stored grains in Africa (Abate et al. 2000).

Identifying the specific pest found within a sample is the first step in controlling insect pests, because insects have different damage potentials, biology, behaviors, growing temperatures, moisture requirements, and reproductive potentials (Mason and McDonough 2012).

Toxic synthetic insecticides being applied in solid and liquid forms against these insect pests are generally accepted as effective but carcinogenic, hazardous to non-target organisms and the environment. There is therefore, the growing interest in adapting naturally existing plant in crop protection to stand the trend of food shortage as a result of insect infestation. In recent years, researchers have been focusing on the secondary compounds of plant extracts to be used as alternatives for chemical insecticides (Adeyemi 2010).

Materials and Methods

Study Area

This study was conducted in the Department of Biology Laboratory, Federal University of Technology Owerri (FUTO) which lies between the coordinates 5.3892° N, 6.9916° E. *Callosobruchus subinnotatus* was identified by an entomologist in the Department of Biology, Federal University of Technology Owerri, Imo State.

Procurement of Experimental Area

The spices (Curry leaf, Scent leaf and Ginger) and Bambara nut were procured at Ihiagwa market, Owerri-west, Imo state. The stored pest *(Callosobruchus subinnotatus)* was procured from infested Bambara nut from Ihiagwa local market.

Experimental Design

The experimental design used was completely randomized design (CRD) replicated four times with four treatments given a total of 16 plates.

Preparation of the Powdered Extracts of Spices

Fresh curry leaf, scent leaf and ginger were collected, washed and chopped up into pieces to facilitate drying and were dried in a well-ventilated area at room temperature for 4 weeks. The dried botanical samples were grinded into powder using mortar and pestle.

Experimental Treatments

The Bambara nut was subjected to three treatments which were replicated 4 times:

T_o = control which includes the Bambara nut grains and *Callosabruchus subinnotatus.*

T_1 = consists of the powdered curry, Bambara nut grains and *Callasobruchus subinnotatus.*

T_2 = consists of the powdered scent leaf, Bambara nut grains and *Callosobruchus subinnotatus.*

T_3 = consists of ginger, Bambara nut grains and *Callosobruchus subinnotatus.*

Parameters /Data collection

Number of Holes

The number of holes bored by the weevil were counted and recorded.

Mortality

The mortality was recorded by counting the dead weevils on daily basis.

Weight Loss

The weight loss was obtained by subtracting the final weight of Bambara nut seeds from the initial weight of Bambara seeds.

Phytochemical Analysis

Phytochemical analysis was carried out on the scent leaf, curry leaf and ginger in the laboratory in order to obtain the phytochemical constituents using standard procedures of Nwachukwu et al. (2018) and Nwachukwu et al. (2020).

Statistical Analysis

Data collected was subjected to analysis of variance procedure ANOVA using SPSS version 20 and the mean difference were determined using least significance difference (LSD) at 0.05% probability level.

Results

Mortality Rate of Bambara Beetle (*Callosobruchus subinnotatus*) on Extracts of Three Spices

The mortality rate was recorded highest on 100g, of ginger powder extract having percentage mortality of 93.33% and was significantly higher than other treatments applied at P=0.05 level. The results also showed that curry leaf powder extracts did not differ significantly in mortality of Bambara nut beetle (Table 1). There was no mortality recorded on control treatment.

Table 1. Effect of powdered extracts of three different spices on Bambara groundnut beetle

Powdered Extracts (g)	Mean number of beetle introduced	Mean number dead	Mean number alive	Mortality (%)
Control 0	$30^a \pm 0.01$	$O^c \pm 0.00$	$30^a \pm 0.01$	0.00
Curry 100	$30^a \pm 0.01$	$ll^b \pm 0.16$	$19^b \pm 1.23$	36.67
Scent 100	$30^a \pm 0.01$	$13^b \pm 0.18$	$17^b \pm 1.20$	43.33
Ginger 100	$30^a \pm 0.01$	$28^a \pm 0.21$	$2^c \pm 0.03$	93.33

Mean along the column having a different superscript of letters differ significantly at P = 0.05

Mean Number of Holes and Weight Loss of Bambara nut Seed

Table 2 shows the results on the mean number of holes and weight loss of Bambara nut seeds treated with different plant spices extract. Ginger powdered extract gave the lower mean number of holes (41) and with lower weight loss of 4.9 g. The Bambara nuts that were not treated with plant spice extracts gave the highest mean number of holes (211) and the highest weight loss 25.5 g of the nuts.

Table 2. Effect of three different spices on Mean number of holes and weight loss of Bambara seeds

Powdered Extract (g)	Initial weight of seeds (g)	Final weight of seeds (g)	Mean number of boles	Weight loss of seeds (g)
Control 0	$250^{a} \pm 0.01$	$224.5^{b} \pm 12.10$	$211^{a} \pm 20.16$	$25.5^{a} \pm 3.23$
Curry leaf 100	$250^{a} + 0.01$	$234.2^{c} \pm 19.12$	$98^{b} \pm 11.0$	$15.8^{b} \pm 2.07$
Scent leaf 100	$250^{a} \pm 0.01$	$239.3^{c} \pm 19.22$	$87^{c} \pm 8.24$	$10.7^{c} \pm 1.62$
Ginger 100	$250^{a} \pm 0.01$	$245.1^{a} \pm 22.14$	$41^{d} \pm 4.35$	$4.9^{d} \pm 0.98$

Mean along the column having different superscript of letters differ significantly at P= 0.05 level.

Phytochemical Screening of Curry Leaf, Scent Leaf and Ginger

The result on proximate composition of curry leaf, scent leaf and ginger powdered extracts shows a high constituents of dry matter (%), followed by Nitrogen free extracts (%), others were ash, crude protein, crude fibre and ether extracts; All expressed in percentages (Table 3). The phytochemical analysis of curry leaf, scent leaf and ginger powdered extracts shows that terpenoids was absent in all. In curry leaf and scent leaf powdered extracts saponins and cardiac glycosides were absent. The most common in the three different spices were flavonoids and alkaloids (Table 4).

Table 3. Proximate composition of curry leaf, ginger and scent leaf

Constituents (%)	Curry leaf	Ginger	Scent leaf
Dry matter	91.01	81.87	89.82
Crude protein	8.68	9.62	8.96
Crude fibre	7.32	10.57	7.87
Ash	10.73	8.16	9.11
Ether extract	4.38	7.33	6.24
Nitrogen free	32.76	42.98	36.43

Table 4. Phytochemical analysis of curry leaf, ginger and scent leaf

Parameters	Curry leaf	Ginger	Scent leaf
Alkaloids	+	+	+
Tannins	++	-	++
Flavonoids	+ +	++	++
Saponins	-	+	-
Terpenoids	-	-	-
Cardiac glycosides	-	+	-

Key: + = Present, - = Absent, ++ = Present in large quantity

Discussion

The present study evaluated the impact of botanical spices powdered extracts in control of Bambara nut pest (*Callosobruchus subinnotatus*). The result showed that mortality rate was recorded highest on 100g of ginger powder extract having percentage mortality of 93.33%. It was followed by scent leaf with a mortality rate of 43:33%. The pesticidal effect of ginger has been studied by Kubra et al. (2013). He reported that ginger and other plants could be used for the control of pest of stored products. The high mortality rate observed in this present study when ginger was used could be as result of the bioactive compounds which were not friendly to *Callosobruchus subtinnotatus.* The result of the photochemical components of ginger is in agreement with the result of research work of Najim (2017) who observed the presence of alkaloids, flavonoids, saponims and cardiac glycosides in varying concentrations. The result from this study also showed that Ginger powdered extract gave the lower mean number of holes and with lower weight loss. The Bambara nuts that were not treated with plant spice extracts gave the highest mean number of holes and the highest weight loss 25.5g of the nuts. Considering this, the plant extracts were effective at controlling the pests at varying degrees.

Conclusions

This study has shown that ginger powdered extract was active against *Callosobruchus subinnotatus* with mortality rate above 90%. The ginger powdered extract was significantly effectively to Bambara nut weevil. The ginger powdered extract reduced grain weight loss, but curry and scent leaf powdered extract had lesser effect.

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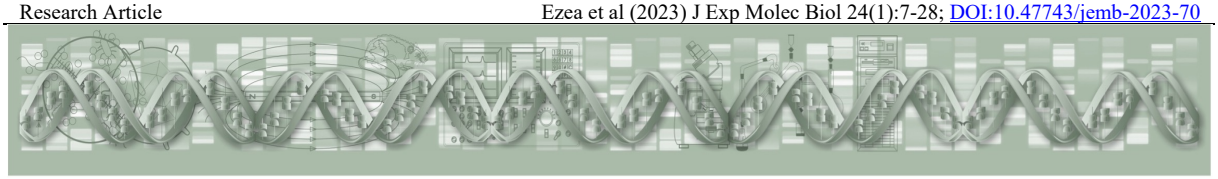

GAMETOCYTE DYNAMICS AND THE EFFECTS OF AQUEOUS AND ETHANOLIC LEAF EXTRACTS OF *LOPHIRA LANCEOLATA* IN MICE INFECTED WITH *PLASMODIUM BERGEI*

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Abstract

Resistance of human malaria parasites to existing chemotherapeutic drugs remains a global challenge to malaria control. This study was an evaluation of the gametocyte dynamics and the potential roles of aqueous and ethanolic leaf extracts of *Lophira lanceolata* in the transmission of *Plasmodium* parasites using mice experimentally infected with *P. berghei* as a model. A total of 192 experimental mice divided into 15 groups in a randomized block design and replicated four times with four mice in each group were used for this study. Thirty-six (36) mice were used for acute toxicity test, 32 mice each for curative antiplasmodial assay, prophylactic assay and gametocyte evaluation, and analyses. Experimental mice were inoculated intraperitoneally with standard inoculum of 1 x 10^7*P.berghei* parasitized red blood cells on first day (DO). Body weights of mice were taken using sensitive digital weighing balance. Blood sample was collected by cardiac puncture using sterile needle and 5ml syringe and preserved in ethylene diaminetetraacetic acid (EDTA) bottle. Haematological components were determined using an autohaematology analyzer SYSMEX KX21. Data analysis was done using student's t-test and one way analysis of variance with multiple comparison tests. Phytochemical screening revealed the presence of phenols, terpenoids, tannins, saponins, alkaloids, flavonoids, steroids, oxalates and cardiac glycosides. The flavonoids had the largest concentrations of 388.24mg/ml, and 350.00mg/ml in ethanolic and aqueous extracts respectively. LD_{50} was found to be $\geq$ 5000mg/kg body weight in both extracts. At $\geq$ 100mg/kg body weight, infected treated mice experienced body weight increases while infected untreated mice experienced decreases in mean body weight from 23.33g to 20.08g (mean 10% weight loss). Aqueous and ethanolic leaf extracts produced variations in number of gametocyte from mice infected with *P.berghei*. On days 7, 14, 21, and 28, the mean gametocytes of the parasite in the control group were 18, 21, 22, and 20 respectively which were significantly ($p < 0.05$) higher compared with the mean gametocytes in the groups of mice treated with aqueous extract, ethanolic extract and chloroquine drugs. The dose level of 10mg/kg body weight of aqueous extract produced the mean gametocyte counts of 10, 10, 8 and 6 on days 7, 14, 21 and 28 respectively which were significantly ($p < 0.05$) lower than the mean gametocyte counts of the control.

Keywords: Gametocyte Dynamics, *Lophira lanceolata*, Mice, Plasmodium Bergei

Introduction

Malaria is a mosquito-borne infectious disease of humans and other animals caused by eukaryotic protists of the genus *Plasmodium*. This genus *Plasmodium* infects mammals, birds, and lizards and is transmitted through the bites of female mosquitoes (*Anopheles* species in

Received 23 August 2022; Accepted 19 January 2023; Published Online 26 January 2023; Published 3 April 2023

mammals or *Culex* species in birds and lizards) [1]. The signs and symptoms of malaria typically begin 8–25 days following infection. However, symptoms of malaria may occur later in those who have taken antimalarial medications as prevention [2]. Initial manifestations of malaria are similar to flu-like symptoms [3] and can resemble other conditions such as sepsis, gastroenteritis, and viral diseases [4]. The presentation may include headache, fever, shivering, joint pain, vomiting, haemolytic anaemia, jaundice, haemoglobin in the urine, retinal damage, and convulsions [5].

In 2015, there were an estimated 438,000 malaria deaths worldwide. Most of these deaths occurred in the African Region (90%), followed by the South - East Asia Region (7%) and the Eastern Mediterranean Region (2%) [6]. Between 2000 and 2015, malaria incidence rates (new malaria cases) fell by 37% globally, and by 42% in Africa. During this same period, malaria mortality rates fell by 60% globally and by 66% in the African Region [6]. Other regions have achieved impressive reductions in their malaria burden. Since 2000, the malaria mortality rate declined by 72% in the Region of the Americas, by 65% in the Western Pacific Region, by 64% in the Mediterranean Region, and by 49% in the South-East Asia Regions [6]. Children under five are particularly susceptible to malaria illness, infection and death. In 2015, malaria killed an estimated 306,000 under five globally, including 292,000 children in the African Region [6]. Between 2000 and 2015, the mortality rate among children under five fell by 65% worldwide and by 71% in Africa [6] According to the latest estimates in 2018, there were an estimated 228 million cases of malaria worldwide; the estimated number of malaria deaths stood at 405 000 [7]. Children aged under 5 years are the most vulnerable group affected by malaria and they accounted for 67% (272 000) of all malaria deaths worldwide [7]. The WHO African Region carries a disproportionately high share of the global malaria burden. Total funding for malaria control and elimination reached an estimated US$ 2.7 billion in 2018 [8]. Contributions from governments of endemic countries amounted to US$ 900 million, representing 30% of total funding [7]. In Nigeria, the burden of malaria is well documented, and has been shown to be a big contributor to the economic burden of disease in communities where it is endemic and is responsible for annual economic loss of 132 billion naira [9]. It is estimated that 300,000 deaths occur each year, and 60% of outpatient visits and 30% hospitalizations are all attributed to malaria [10]. About 50% of the population has at least one episode of malaria annually resulting in high productivity losses [10] The disease is particularly virulent among pregnant women and children under 5 years of age due to their low levels of immunity [11]. The trend is rapidly increasing due to the current malaria resistance to first line antimalarial drugs [11] It is responsible for over 90% of reported cases of tropical disease in Nigeria [11]. Five species of *Plasmodium* can infect humans. Severe disease is largely caused by *Plasmodium falciparum* while the disease caused by *Plasmodium ovale* and *Plasmodium malariae* are generally a mild disease and is rarely fatal [12]. *Plasmodium falciparum* is the most predominant parasite species accounting for about 98% of malaria cases [13]. In Nigeria, malaria transmission occurs all year round in the South, and is more seasonal in the North. The country accounts for a quarter of all malaria cases in the WHO African Region [6].

Malaria transmission can be reduced by preventing mosquito bites by distribution of mosquito nets and insect repellants, or by mosquito-control measures such as spraying insecticides and draining standing water (where mosquitoes breed). The challenge of producing a widely available vaccine that provides a high level of protection for a sustained period is still to be met, although several vaccines are under development [14]. Presently, vector control is the mainstay to prevention and reduction of malaria transmission. Two forms of vector control are effective in a wide range of circumstances. They are insecticide-treated mosquito nets (ITNs) and indoors residual spraying (IRS). Over the last 15years, there has been a major increase in coverage of ITNs in Sub-Sahara Africa. By 2014, more than half (56%) of the population had access to an ITN, compared to less than 2% in 2000 [6]. In 2014, 166 million people globally were protected

by indoor residual spraying (IRS), including 50 million people in Africa. An estimated 663 million cases of malaria have been averted in Sub-Saharan Africa since 2001 as a direct result of the scale-u p of three (3) key interventions: Insecticide - treated mosquito nets (ITNs), artemisinin-based combination therapy (ACTs) and indoors residual spraying (IRS) (Bhatt *et al*., 2015). It is estimated that 69% of the 663 million fewer malaria cases attributable to interventions were due to the use of mosquito nets, 21% due to ACTs and 10% due to indoor spraying [6]. Across Africa, the prevention of new cases of malaria attributable to malaria control activities saved an estimated US $900 million in case management costs between 2001 and 2014 [6]. Despite substantial costs savings, malaria has placed a heavy economic burden on health systems in Africa. Since 2000, the average annual cost of case management alone is estimated at nearly US $300 million [6]. The efficacy of the conventional drugs against malaria parasite has been reported with variable successes [15]. The toxic effects of these chemicals on humans, the development of resistance to it by target parasites and the high cost of drugs have paved way for herbal remedies as reasonable alternative [16].

The continuous spread of *P. falciparum* resistance to antimalarial drugs possess serious threat to malaria control programmes. In Nigeria, a nationwide surveillance data on drugs efficacy showed that Chloroquine (CQ) and Sulphadoxine-pyrimethamine (SP) are no longer viable therapeutic options for the effective treatment of human malaria [17]. Although vaccines could be the best long term control option, they are still undergoing clinical trials. This in addition to the increased number of drug- resistant parasites, makes the development of novel antimalarial urgent. The high cost of malaria treatment has left the poor masses of Nigeria heavily reliant on traditional practitioners and medicinal plants for the treatment of the disease [8].

Numerous plants indigenous to Nigeria have been found with amazing antimalarial properties. It is therefore highly essential that indigenous plants used by the people to treat malaria be scientifically investigated to prove their ethno-therapeutic use [18]. Plants have always been considered to be a possible alternative and rich source of new drugs. The search for malaria remedies in plants and improved interest in plant drugs by many communities staying in endemic area led to the choice using *Lophira lanceolata* plant in establishing the scientific basis for the treatment of malaria. Phytochemical screening of *Lophira lanceolata* leaves and seeds revealed the presence of compounds such as flavonoids, anthraquinones, phenols, saponins and tannins [19]. *Lophira lanceolata* has a range of pharmacological effects. The plant has been found to possess antioxidant, antimalarial, anti-hypertensive effect, antibacterial, antiviral and sexual enhancement properties [20]. Besides the efficacy of herbal remedies, there are always serious concerns for their safety. Some researchers have earlier reported the safety of an aqueous stem bark extract of *Lophira lanceolata* in Sprague dawley rats [21].

Materials and Methods

Plant Collection and Authentication

The fresh leaves of *Lophira lanceolata* were collected in July 2017 at Obinze, Owerri West Local Government Area, Imo State, Nigeria. The plant materials were transported in polythene bags to the Research Laboratory of Department of Biology, Federal University of Technology Owerri, Imo State for identification. The specimen was identified and authenticated by Dr. C. M. Duru, a plant taxonomist in the Department of Biology, Federal University of Technology Owerri, Imo State, Nigeria. Voucher specimen number was left in the Departmental herbarium

Study Area

Preparation and administration of leaf extracts

Fresh leaves of the plant were sorted to remove any dead matter and other unwanted particles. The plant leaves were washed in tap water and air-dried for two (2) weeks [22]. The crude extracts were prepared by cold maceration technique according to [21].

Ethanol extraction

One hundred grams (100g) of the air-dried powdered leaf were weighed and mixed with ethanol 95% (500 ml) using a rotary shaker at 200 rpm for 24 hours at room temperature (25 ± 3^0C). The filtrates were pooled and filtered two times on cotton wool and once on Whatman (No 1) filter paper. The ethanol was evaporated at 50^0C using a rotary evaporator [2]. The prepared extract was weighed, labeled and stored at 4^0C in air tight bottles until ready for use.

Aqueous extraction

One hundred grams (100 g) of leaf powder was dissolved in 500 ml of distilled water. The solvents were evaporated using a rotary evaporator at 100 rpm. The prepared extract was weighed, labeled and stored at 4^0C in air tight bottles until ready for use. The yields of the extract obtained were calculated by the formula as shown below:

$$\text{Extraction yield} = \frac{\text{Weight of concentrated extract}}{\text{Weight of plant dried powder}} \times \frac{100}{1}$$

The weighed extracts were administered orally to animals using a metal orpharyngeal cannula.

Phytochemical screening

The aqueous and ethanolic leaf extracts were screened for bioactive ingredients such as terpenoids, flavonoids, steroids, cardiac glycosides, alkaloids, tannins, phenols, oxalate, and saponins using standard procedures [20]. The change in coloration determined the presence or absence of a particular bioactive parameters investigated.

Qualitative phytochemical analysis

Qualitative phytochemical analysis of aqueous and ethanolic extracts of *Lophira lanceolata* was conducted following the standard procedures as described by [19].

Test for Alkaloids (Wagner's reagent)

Added 4 ml of extract was treated with 4 drops of Wagner's reagent [1.27 g of iodine and 2 g of potassium iodide in 100 ml of water] and observed for the formation of reddish brown precipitate (or colouration).

Test for Cardiac glycosides (Keller Kelliani's test)

Added 5 ml of leaf extract to 2 ml of glacial acetic acid in a test tube and a drop of ferric chloride solution was added to it. This was carefully underlayed with 1ml concentrated sulphuric acid. A brown ring at the interface indicated the presence of deoxysugar. A violet ring appeared below the ring while in the acetic acid layer, a greenish ring formed.

Test for Flavonoids (Alkaline reagent test)

Added 2 ml of leaf extract to 4 drops of 20% sodium hydroxide solution in a test tube. Formation of intense yellow colour, which became colourless on addition of dilute hydrochloric acid, indicated the presence of flavonoids.

Test for Phenols (Ferric chloride test)

Added 2 ml of the extract to aqueous 5% ferric chloride in a test tube and observed for formation of deep blue or black colour.

Test for Saponins (Foam test)

To 2 mls of extract was added to 6 ml of water in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam that confirms the presence of saponins.

Test for Steroids (Liebermann-Burchard test)

To 1 ml of extract in a test tube was added 3 drops of chloroform, acetic anhydride and concentrated sulphuric acid (H_2SO_4) and observed for the formation of dark pink or red colour.

Test for Tannins (Braymer's test)

To 2 mls of extract in a test tube was added 4 drops of 10% alcoholic ferric chloride solution and observed for formation of blue or greenish colour solution.

Test for Terpenoids (Salkowki's test)

To 1 ml of chloroform in a test tube was added to 2 ml of each extract followed by 3 drops of concentrated sulphuric acid and observed for formation of a reddish brown precipitate.

Test for Oxalate

To 3 ml of extract in a test tube was added 3 drops of ethanoic acid glacial and observed for formation of a greenish black colouration.

Quantitative phytochemical analysis

The phytochemicals which were present in the ethanol and aqueous extracts of *Lophira lanceolata* were determined and quantified by standard procedures [19].

Tannins Determination by titration

The Folling Dennis titrating method as described by Pearson (1976) was used. To 20 g of the crushed sample in a conical flask was added 100 ml of petroleum ether and covered for 24hrs.The sample was then filtered and allowed to stand for 15 minutes for petroleum ether to evaporate. It was then re-extracted by soaking in 100 ml of 10% acetic acid in ethanol for 4 hours. The sample was then filtered. 25 ml of ammonium hydroxide (NH_4OH) was added to the filterate to precipitate the alkaloids. The alkaloids were heated with electric hot plate to remove some of the NH_4OH still in solution. The remaining volume was measured and 5ml of this was added to 20ml of ethanol. It was titrated with 0.1 M soium hydroxide (NaOH) using phenolphthalein as indicator until pink end point was reached. Tannin content was then calculated in percentage molarity of sample analyzed.

Determination of saponins

Added 5 g of the sample to 4 drops of 20% acetic acid a test tube and allowed to stand in a water bath at 50^0C for 24 hours. This was filtered and the extract was concentrated to one-quarter of the volume, using a water bath. Concentrated NH_4OH was added drop-wise to the extract until the precipitate was complete. The whole solution was allowed to settle and the precipitate was collected by filtration and weighed. The saponin content was weighed and calculated in percentage of sample analyzed.

Determination of alkaloids

The alkaloids content of the extract was determined using standard procedures as described by [23]. Five grams (5g) of the sample was weighed into a 250 ml beaker and 200 ml of 20 % acetic acids in ethanol was added, covered and allowed to stand for 4hours at 25^0C.This was filtered with filter paper No 42 and the filtrate was concentrated using a water bath (Mammert), to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was collected and washed with dilute NH_4OH (1% ammonia solution). Then the solution was filtered with pre-weighed filter paper. The residue on the filter paper was the alkaloid which was dried in the precision oven at 800 ^{0}C. The alkaloid content was calculated and expressed as a percentage of the weight of the sample analyzed.

Determination of steroids

Added 1ml of steroid solution into 10 ml volumetric flasks. Sulphuric acid (4 N, 2 ml) and iron (iii) chloride (0.05% W/N, 2ml) were added followed by potassium hexacyanoferrate (iii) solution (0.5% W/N, 0.05ml) at 70 ± 20^0C for 30 minutes with occasional shaking and diluted to 8ml mark with distilled water. The absorbance was measured at 780nm against the reagent blank.

$$\text{Steroids} = \frac{\text{Concentration of Standard x Absorbance of sample}}{\text{Absorbance of standard x Sample weight (g)}}$$

Determination of cardiac glycosides

To 1ml of extract in a test tube was added 1 ml of 2% solution of 3.5-DNS (Dinitrosallcyclic acid) in methanol and 1 ml of 5% aqueous NaOH. It was boiled for 20 minutes (until brick -

red precipitate was observed) and the boiled sample was filtered. The weight of the filter paper was weighed before filtration. The filter paper with the absorbed residue was dried in an oven at 50^0C till dryness and the weight of the filter paper with residue was noted. The cardiac glycoside was calculated in percentage.

Determination of flavonoids

Total flavonoid content was determined by aluminium chloride method using a catechin as a standard. Added 1 ml of test sample and 4 ml of water to a volumetric flask (10 ml). After 5 minutes, 0.3 ml of 5% sodium nitrate and 0.3 ml of 10% aluminum chloride were added. After 6 minutes incubation at room temperature, 2 ml of 1 ml of NaOH was added to the reaction mixture. Immediately the final volume was made up to 10 ml with distilled water. The absorbance of the reaction mixture was measured at 510 nm against a blank spectrophotometrically.

Determination of terpenoid

The total terpenoid content in the aqueous and ethanolic extract of *Lophira lanceolata* were determined by the method as described by Ferguson (1956). Pippete1g of the plant extract into a conical flask and soaked in ethyl alcohol (70%) (10 ml) for one day. Then it was filtered and the filtrate was extracted with petroleum ether. The ether extract was taken as the measure of total terpenoid.

Determination of oxalate by titration method

This was determined according to [24]. This determination involved three major steps: digestion, oxalate precipitation and permanganate titration.

Digestion:

a) Suspended 2g of sample was in 100ml of distilled water in a 250 ml volumetric flask.
b) Added 10 ml of 0.1M HCl to the suspension and digested at 100^0C for 1hour.
c) Cooled the filtrate and then made up to 250ml mark with distilled water before titration.

Oxalate Precipitation:

Duplicated portions of 125 ml of the filtrate were measured into beakers and four drops of methyl red indicators added. This was followed by the addition of NH_4OH solution (dropwise) until the test solution changed from pink colour to faint yellow colour (pH 4-4.5). Each portion was then heated 90^0C, cooled and filtered to remove precipitate containing ferrous ions. The filtrate was again heated to 90^0C and 10 ml of 5% Calcium Chloride ($CaCl_2$) solution was added while being stirred constantly. After heating, it was cooled and left overnight at 25^0C. The solution was then centrifuged at 2500 rpm for 5 minutes. The supernatant was decanted and the precipitate completely dissolved in 10 ml of 20% (v/v) H_2SO_4 solution.

Permanganate Titration

At this point, the total filtration resulting from digestion of 2 g of sample was made up to 300ml aliquots of 125 ml of the filtrate was heated until near boiling and then titrated against 0.05M standard KMnO4 solution to faint pink colour which persisted for 30 seconds. The calcium oxalate content was calculated using the formular:

$$\text{Oxalate content} = \frac{T \times (Vme) \times (Df)}{(ME \times Mf)} \times (Mg/100g)$$

where T was the titre of KMnO4 (ml), Vme was the volume mass equivalent (ie 1 ml of 0.05M solution solution is equivalent to 0.00225 g anhydrous oxalic acid). Df is the dilution factor (Vt/A), Vt the total volume of titrate (300 ml) and A is the aliquot used (250 ml), ME is the molar equivalent of KMnO4 in oxalate and MF is the Mass of sample used.

Determination of phenol

The quantity of phenols was determined using the spectrophotometer method. Boiled 2 g of the plant sample with 50 ml ethanol for 50 minutes. Pippetted 5 ml of the boiled sample into 50 volumetric flask and 10 ml of distilled water was added. After the addition of distilled water, 2

ml of NH_4OH solution and 5 ml of concentrated pentanol was added to the mixture. The sample was made up to the 25 ml mark with distilled water and left for 30 minutes to react for colour development and measured at 505 nm wavelength using a spectrophotometer.

$$\text{Phenol Content} = \frac{\text{Concentration of Standard x Absorbance of sample}}{\text{Absorbance of standard x Sample weight (g)}}$$

Parasite collection

A strain of *Plasmodium berghei* that is chloroquine sensitive was obtained from donor-infected mice maintained at Animal Facility Centre, Faculty of Veterinary Medicine, University of Nigeria, Nsukka

Experimental animals

Male Swiss albino mice were obtained from the Department of Biochemistry, Federal University of Technology, Owerri, Imo State, Nigeria. The mice were allowed to acclimatize to the laboratory environment under ambient temperature of 26 - 32ºC and humidity for at least three days before being subjected to the experiments (Peter and Anatoli 1998). The animals were housed in cages and fed with growers mash (Vital feeds) at the Department of Biology, Federal University of Technology, Owerri. All the mice were given free access to food and water ad libitum throughout the experimental period. Also, good hygiene was maintained by regular cleaning and removal of feaces and spilled feed from cages occasionally.

Inoculum preparation

The donor mice were monitored for signs of infection such as anorexia, ruffled appearance, shivering, heat-seeking behaviour and lethargy. Blood samples were taken from the second day to confirm the extent of parasitaemia in the donor mice. The mice were anaesthetized in a glass jar containing cotton wool soaked in chloroform. Blood were collected from the donor mice by cardiac puncture using 5ml sterile syringes and needles. The blood samples were diluted with Trisodium citrate (TC) medium so that each 0.2ml contain approximately $1.0x10^7$ infected red cells [20].

Acute toxicity tests

The aqueous and ethanolic extracts of *Lophira lanceolata* leaves were evaluated for their toxicity in *P. berghei* non-infected male Swiss albino mice using modified Lorke's method [25] method of determining toxicity level of extract in mice. The study for each leaf extract was carried out in two phases. In phase one of the study, nine mice were randomized into three groups of three mice each and were given 10, 100, and 1000mg/kg body weight of the extract orally. The mice were observed for signs of toxicity which included but not limited to salivation, stretching of the entire body, weakness, sleep, respiratory distress, coma and death in the first four hours and subsequently daily for seven days. In the second phase of the study another fresh set of nine mice were randomized into three groups of three mice each and were given 1600, 2900 and 5000 mg/kg body weight of the extract orally based on the result of the first phase. These were observed for signs of toxicity and mortality for the first critical four hours and thereafter daily for seven days. The LD_{50} were calculated as the square root of the product of the lowest lethal dose and highest non-lethal dose i.e., the geometric mean of the consecutive doses for which 0 and100% survival rates were recorded in the second phase. The oral median lethal dose was calculated using the formula [26].

$$LD_{50} = \sqrt{(\text{Minimum toxic dose x maximum tolerated dose})}$$

Determination of Parasitaemia

Thin film from tail blood sample was prepared by placing a drop of blood on a microscope slide and thereafter used a second slide held at an angle to spread the blood and pulled it across the slide, forming a "feathered edge" consisting of a single layer of cells at the end of the smear [26]. The thin blood film was allowed to air dry at room temperature before staining with 10%

Giemsa at pH 7.2 for 10 minutes. The blood film was rinsed in distilled water (and back of the slide carefully cleaned) and air dried to remove particles that might obstruct the film examination. Slides were viewed using a compound microscope with oil immersion (x100 magnification). The percentage parasitaemia was obtained by counting the number of parasitized red blood cells out of 500 erythrocytes in random fields of the microscope Percentage parasitaemia and the average chemosuppression were calculated using the formula below:

$$\% \text{ parasitemia} = \frac{\text{No of parasitised RBC}}{\text{Total No of RBC counted}} \times \frac{100}{1}$$

Average percentage chemosuppression was calculatd as $\frac{A - B}{A} \times \frac{100}{1}$

Were A is the average percentage parasitaemia in negative control group and B average percentage parasitaemia in the test group.

Curative antiplasmodium assay

To assess the schizonticidal activity of either aqueous or ethanolic extracts, 32 mice were inoculated intraperitoneally with standard inoculum of $1x10^7$ *Plasmodium berghei* parasitized red blood cells on the first day (D_0). 72 hours later, the mice were randomized into 8 groups of 4 animals per group. Group 1 received 10ml/kg/day of distilled water served as a negative control group. Groups 2, 3, and 4 animals were administered with 10, 100 and 200mg/kg/day of the aqueous extract orally respectively. Groups 5, 6, and 7 animals were administered with 10, 100 and 200mg/kg/day of the ethanolic extract orally respectively. Group 8 animals were administered with 5mg/kg/day of chloroquine as a positive control group. All the drugs were administered to the animals once daily for 5 days.

Evaluation of gametocyte dynamics

To evaluate the dynamics of gametocyte production of infected mice treated with either aqueous or ethanolic extracts, 32 mice were inoculated intraperitoneally with standard inoculum of $1x10^7$ *Plasmodium berghei* parasitized red blood cells on the first day (D_0). 72 hours later, the mice were randomized into 8 groups of 4 animals per group. Group 1 received 10ml/kg/day of distilled water as a negative control group. Groups 2, 3, and 4 animals were administered with 10, 100 and 200mg/kg/day of the aqueous extract orally respectively. Groups 5, 6, and 7 animals were administered with 10, 100 and 200mg/kg/day of the ethanolic extract orally respectively. Group 8 animals were administered with 5mg/kg/day of chloroquine served as the positive control group. All the drugs were administered to the animals once daily for 28 days. Thin films from tail blood samples obtained on Day 7, Day 14, Day 21 and Day 28 were examined microscopically to monitor gametocytaemia level. On 29th Day, thin film from tail blood sample of each mouse was prepared. The percentage gametocytaemia was obtained by counting the number of red blood cells (RBC) having gametocytes in every 500 erythrocytes in random fields of the microscope

Percentage gametocytaemia was calculated using the formula below:

$$\% \text{ gametocytaemia} = \frac{\text{No of RBC infected with gametocytes}}{\text{Total No of RBC counted}} \times \frac{100}{1}$$

Data Analysis

The data was analysed using the Statistical Package for Social Sciences (SPSS) version 17. T-test and and one way analysis of variance (ANOVA) with multiple comparison tests were used to compare parameters.

Results

Extract Yield

The aqueous and ethanolic leaf extracts of *L. lanceolata* yielded 17.43g (17.43%) and 36.17g (36.17%) respectively (Table 1).

Table 1. Yield of aqueous and ethanolic crude leaf extract of *L. lanceolata*

Solvent	Plant powder (g)	Extraction solvent (ml)	Yield (g)	Yield (%)
Aqueous	100	500	36.17	36.17*
Ethanolic	100	500	17.43	17.43*

Legend: ml: milliliter; g: gram; %: percentage; ***:** there was significant difference between these values ($p< 0.05$).

Phytochemical Screening

The results of the qualitative phytochemical analysis of aqueous and ethanolic leaf extracts of *L. lanceolata* showed the presence of tannins, saponins, alkaloids, flavonoids, phenols, steroids, terpenoids, oxalate, and cardiac glycosides (Table 2).

Table 2. Qualitative analysis of ethanolic and aqueous extracts of *Lophira lanceolata*

S/N	Parameters	Ethanolic extract	Aqueous extract
1	Tannins	Present	Present
2	Saponins	Present	Present
3	Alkaloids	Present	Present
4	Flavonoids	Present	Present
5	Cardiac glycosides	Present	Present
6	Phenols	Present	Present
7	Steroids	Present	Present
8	Terpenoids	Present	Present
9	Oxalate	Present	Present

The results of quantitative phytochemical analysis showed that the flavonoid content had the highest value in both ethanolic leaf extract (388,240 mg/ml) and aqueous leaf extract (350.00 mg/ml). Of all the phytochemical constituents analysed, steroids was the lowest in both aqueous and ethanolic leaf extract of *L. lanceolata* (Table 3).

Table 3. Quantitative analysis of ethanolic and aqueous extracts of *Lophira lanceolata*

S/N	Parameters	Ethanolic extract (mg/ml)	Aqueous extract (mg/ml)
1	Tannins	$35.50^{e} \pm 2.05$	$22.70^{e} \pm 1.72$
2	Saponins	$0.038^{g} \pm 0.02$	$0.030^{h} \pm 0.01$
3	Alkaloids	$124.00^{d} \pm 12.03$	$69.00^{c} \pm 4.11$
4	Flavonoids	$388.24^{a} \pm 19.14$	$350.00^{a} \pm 17.16$
5	Cardiac glycosides	$188.00^{c} \pm 14.11$	$97.00^{b} \pm 8.17$
6	Phenols	$0.04^{g} \pm 0.02$	$0.11^{g} \pm 0.07$
7	Steroids	$0.02^{h} \pm 0.00$	$0.01^{i} \pm 0.00$
8	Terpenoids	$0.40^{f} \pm 0.10$	$0.73^{f} \pm 0.30$
9	Oxalate	$294.75^{b} \pm 16.23$	$29.25^{d} \pm 1.96$

Values are mean ± S. D.Mean values having different superscripts along the same column are significantly different ($P<0.05$)

Acute Toxicity Test

The result of the acute toxicity study indicated that none of the different doses of extract (10 – 5000mg/kg body weight of the extract) in all the groups and phases caused mortality of mice for over ten days (Table 4). The behavioural signs of toxicity observed in mice given 100mg/kg body weight and above included stretching, salivation, and reduced activity. The median lethal dose (LD_{50}) was found to be ≥ 5000mg/kg body weight.

Table 4. LD_{50} determination of aqueous and ethanolic leaf extract of *L. lanceolata*

Phases	Aqueous Number of mice (ratio)	Ethanolic Number of mice (ratio)
Phase one		
Dosed (mg/kg)		
10	$3(^0/_3)$	$3(^0/_3)$
100	$3(^0/_3)$	$3(^0/_3)$
1000	$3(^0/_3)$	$3(^0/_3)$
Phase two		
Dosed (mg/kg)		
1600	$3(^0/_3)$	$3(^0/_3)$
2900	$3(^0/_3)$	$3(^0/_3)$
5000	$3(^0/_3)$	$3(^0/_3)$

Effects of Aqueous and Ethanolic Leaf Extracts of *Lophira lanceolata* on Gametocyte Production

The oral administration of aqueous and ethanolic leaf extract of *Lophira lanceolata* on mice infected with *Plasmodium berghei* produced variations in number of the gametocyte production (Table 5). On day 7, 14, 21, and 28, the mean gametocytes of the parasite in the control group were 18, 21, 22, and 20 respectively which were significantly ($p< 0.05$) higher compared with the mean gametocytes in the groups of mice treated with aqueous extract, ethanolic extract and chloroquine drugs. The dose level of 10mg/kg body weight of aqueous extract produced the mean gametocyte counts of 10, 10, 8 and 6 on Day 7, Day 14, Day 21 and Day 28 respectively which were significantly ($p < 0.05$) lower than the mean gametocyte counts of the control. The mice treated with chloroquine at 5mg/kg body weight showed a significant decrease in gametocyte count ($p < 0.05$) on Day 7, Day 14, Day 21 and Day 28 compared with the three different dose levels of aqueous and ethanolic extracts. There were significant differences ($p < 0.05$) in mean gamatocytes counts at dose levels of 10mg/kg body weight, 100mg/kg body weight, 200mg/kg body weight of aqueous extract. However, there were no significant difference at dose levels of 10mg/kg body weight, 100mg/kg body weight and 200mg/kg body weight of ethanolic extract.

The percentage gametocytaemia of *P. berghei* of mice in the negative control group were 3.60%, 4.10%, 4.35%, and 4.05% on Day 7, 14, 21 and 28 respectively (Figure 1). The dose level of 100mg/kg body weight of aqueous extract of *L. lanceolata* recorded low percentage gametocytaemia of 0.70% on Day 28. The lowest percentage gametocytaemia (0.10%) was recorded in the positive control group of mice on Day 7 while the highest percentage gametocytaemia (4.35%) was recorded in the negative control group of mice on Day 21.

Table 5. Effects of aqueous and ethanolic leaf extracts of *Lophira lanceolata* and chloroquine on gametocyte production of *P. berghei* in mice

Treatments (mg / kg)	Mean Gametocyte Count in Mice in Days			
	Day 7	Day 14	Day 21	Day 28
Distilled water treated				
10	$18^{a} \pm 1.53$	$21^{a} \pm 1.57$	$22^{a} \pm 1.59$	$20^{a} \pm 1.56$
Aqueous extract treated				
10	$10^{de} \pm 0.40$	$10^{d} \pm 0.40$	$8^{c} \pm 0.35$	$6^{c} \pm 0.31$
100	$11^{d} \pm 0.41$	$12^{d} \pm 0.43$	$7^{c} \pm 0.33$	$4^{d} \pm 0.25$
200	$15^{c} \pm 1.48$	$16^{c} \pm 1.50$	$12^{b} \pm 0.43$	$8^{b} \pm 0.35$
Ethanolic extract treated				
10	$16^{bc} \pm 1.50$	$16^{c} \pm 1.50$	$10^{bc} \pm 0.40$	$6^{c} \pm 0.31$
100	$17^{b} \pm 1.52$	$19^{b} \pm 1.54$	$11^{b} \pm 0.41$	$6^{c} \pm 0.31$
200	$12^{d} \pm 0.43$	$15^{c} \pm 1.48$	$3^{d} \pm 0.22$	$4^{c} \pm 0.25$
Chloroquine treated				
5	$9^{e} \pm 0.38$	$7^{e} \pm 0.33$	$1^{d} \pm 0.13$	$1^{e} \pm 0.13$

Values are mean ± S. D n = 4. Mean values having different superscripts along the same column are significantly different ($P<0.05$)

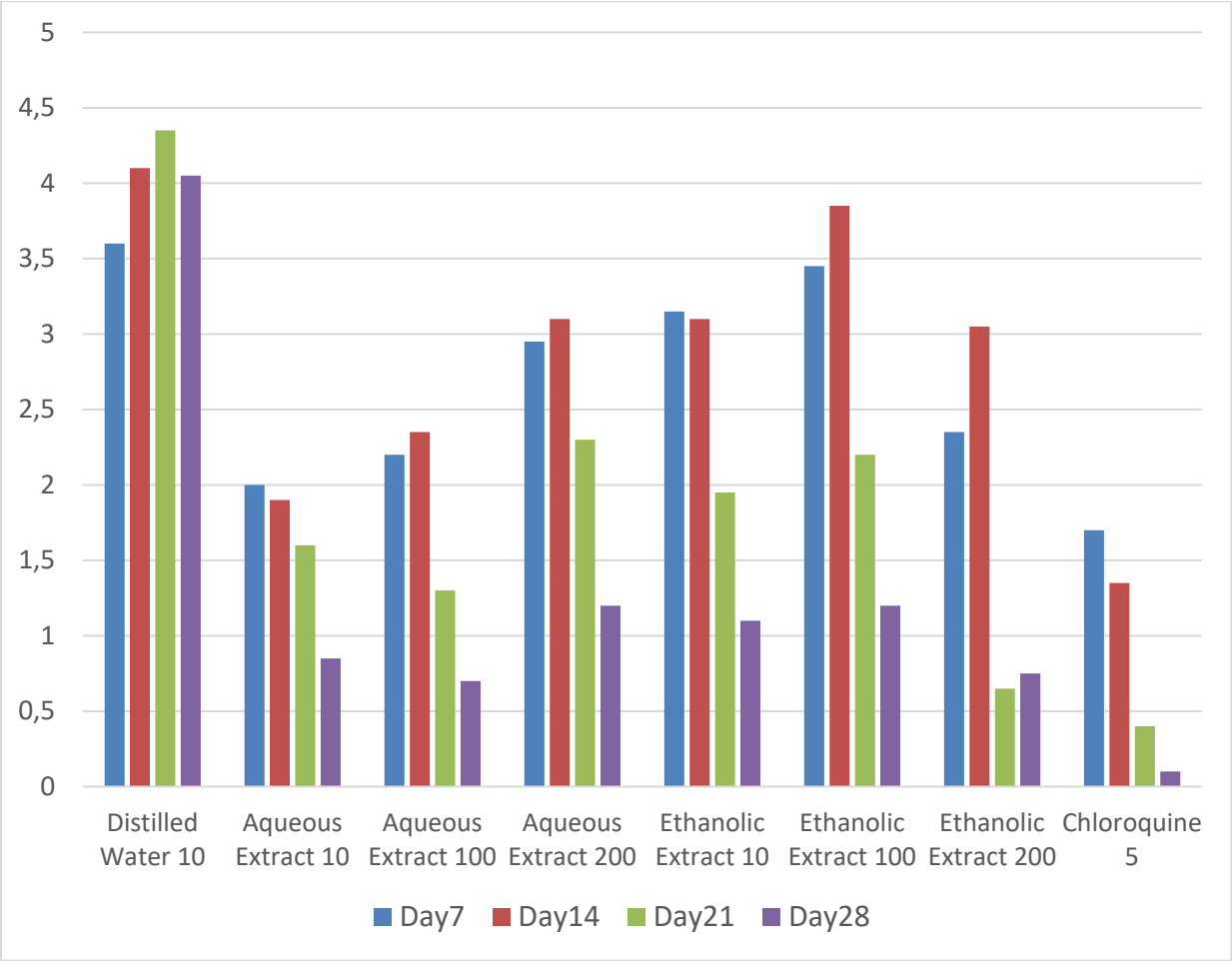

Figure 1. Percentage gametocytaemia of *P. berghei* of mice in days

Prophylactic Effect of Aqueous and Ethanolic Leaf Extract of *Lophira lanceolata* and chloroquine against *P. berghei* infection in mice

The aqueous leaf extract of *Lophira lanceolata* exerted significant (<0.05) non – dose - dependent reduction in level of parasitaemia with chemosuppressions of 48.79%, 24.26%, 29.38% and 80.32% at 10mg/kg, 100mg/kg, 200mg/kg body weight and the chloroquine treated groups respectively (Table 6). Also the ethanolic leaf extract of *Lophira lanceolata* exhibited significant ($p < 0.05$) non – dose - dependent reduction in levels of parasitaemia with chemosuppressions of 43.94%, 35.74%, 22.10% and 80.32% at 10mg/kg, 100mg/kg, 200mg/kg and 5mg/kg the chloroquine treated groups respectively.

Table 6. Prophylactic Effects of aqueous and ethanolic leaf extracts of *L. lanceolata* and chloroquine against *P. berghei in mice*

Test substances	Dose mg/kg	% parasitaemia	% inhibition
Distilled water treated	10	$18.55^{a} \pm 0.29$	0.00 ±0.00
Aqueous extracts treated	10	$9.50^{d} \pm 0.19$	48.79 ±1.58
	100	$14.05^{b} \pm 0.23$	24.26±24.26
	200	$13.05^{c} \pm 0.22$	29.38 ±0.91
Ethanolic extract treated	10	$10.40^{d} \pm 0.15$	43.94 ±1.41
	100	$12.25^{c} \pm 0.18$	35.74 ±1.02
	200	$14.45^{b} \pm 0.26$	22.10 ±0.67
Chloroquine treated	5	$3.65^{e} \pm 0.09$	80.32 ±2.00

($P<0.05$) Each result is a mean of 4 mice ± controls

Curative effect of aqueous and ethanolic leaf extract *Lophira lanceolata* and chloroquine against *P. berghei* infection in mice

The aqueous and ethanolic leaf extract of *Lophira lanceolata* showed significant dose dependent antiplasmodial activity at the various concentrations. The dose level of 10mg/kg, 100mg/kg, 200mg/kg body weight of aqueous extract and the 5mg/kg chloroquine showed chemosuppressions of 12.09%, 20.60%, 60.44% and 89.56% respectively (Table 7).

Moreso, the dose level of 10mg/kg, 100mg/kg, 200mg/kg body weight of the ethanolic extract and 5mg/kg of the chloroquine drug showed chemosuppression of 12.64%, 17.31%, 41.21% and 89.56% respectively.

Table 7. Curative Effects of aqueous and ethanolic leaf extracts of *L. lanceolata* and chloroquine against *P. berghei in mice*

Test substances	Dose mg/kg	% parasitaemia	% inhibition
Distilled water treated	10	18.20^{a}± 0.53	0.00 ±0.00
Aqueous extracts treated	10	16.00^{b} ± 0.50	12.00 ±0.49
	100	14.45^{c} ± 0.48	20.60 ±0.61
	200	7.20^{e} ± 0.34	60.44 ±1.78
Ethanolic extract treated	10	15.90^{b} ± 0.49	12.64 ±0.43
	100	15.05^{c} ± 0.48	17.31 ±0.4
	200	10.70^{d} ± 0.41	41.21 ±1.38
Chloroquine treated	5	1.90^{f} ± 0.17	89.56 ±2.11

(P<0.05) Each result is a mean of 4 mice ± controls

Mean body weight of mice

The mean body weight of the mice in the negative and positive control groups were 22.53g and 22.60g respectively at the commencement of the study (Table 8). The mean body weight for the negative and positive control groups increased to 23.33g and 23.63g at the end of the experimental period, indicating a weight gain of 3.55% and 4.56% respectively. The infected untreated mice had a decrease in mean body weight from 23.33g to 20.08g, showing a 10.08% weight loss. The infected mice treated with aqueous extract at the dose levels of 10mg/kg body weight, 100mg/kg body weight and 200mg/kg body weight had the mean weight of between 23.68 -24.53g at the beginning of the experiment to between 24.28 -24.93g at the end of the treatment indicating 2.53%, 0.61% and 2.17% weight gains at the three dose levels respectively. The uninfected mice treated with aqueous extract at the dose level of 200mg/kg body weight had Mean weight of 26.63g at the beginning of the experiment and 26.88 at the end of the experimental period resulting in 0.95% weight gain.

Table 8. Mean body weight of mice before and after treatment with *L. lanceolata* aqueous leaf extract

Treatments groups (mg / kg)	Mean weight (g)		
	Day 0	Day 28	% change
Negative control			
10	22.53^{c}± 0.59	23.33^{b} ± 0.60	3.55 ±0.27
Infected, untreated			
0	22.33^{c} ± 0.58	20.08^{c} ± 0.56	-10.08 ±0.05
Infected, aqueous extract treated			
10	23.68^{c} ± 0.61	24.28^{b} ± 0.62	2.53 ±0.14
100	24.53^{b} ± 0.63	24.68^{b} ± 0.64	0.61 ±0.07
200	24.40^{b} ± 0.62	24.93^{b} ± 0.65	2.17 ±0.10
Uninfected, aqueous extract treated			
10	25.48^{a} ±0.63	26.43^{a} ± 0.67	3.73 ±0.23
100	26.43 ^{a}±0.64	27.05^{a} ± 0.69	2.35 ±0.12
200	26.63^{a} ± 0.65	26.88^{a} ± 0.68	0.95 ±0.04
Infected chloroquine treated			
5	22.60^{c} ± 0.60	23.63 ^{b}± 0.61	4.56 ±0.38

Values are mean ± S. D n = 4. Mean values having different superscripts along the same column are significantly different ($P < 0.05$).

The oral administration of ethanolic leaf extract of *L. lanceolata* caused some changes in the weight of the individual mice subjected to the experiment (Table 9). The dose level of 200mg/kg body weight of the ethanolic extract in infected mice caused an increase in body weight of the mice from 26.35g at the beginning of the experiment to 27.13g at the end of the experiment resulting in 2.96% weight gain. The mean weight of the uninfected mice treated with ethanolic extract at the dose levels of 10mg/kg body weight,100mg/kg body weight and 200mg/kg body weight caused 6.44%, 3.62% and 3.81% weight gains respectively.

Table 9. Mean body weight of mice before and after treatment with *L. lanceolata* ethenolic leaf extract

Treatments groups (mg / kg)	Mean weight (g)		
	Day 0	Day 28	% change
Negative control			
10	22.53^{e} ± 0.59	23.33^{d} ± 0.60	3.55 ±0.27
Infected, untreated			
0	22.33^{e} ± 0.58	20.08^{e} ± 0.56	-10.08 ±0.05
Infected, ethanolic extract treated			
10	27.25^{a} ± 0.71	27.75^{a} ± 0.73	1.83 ±0.08
100	24.30^{d} ± 0.62	24.93^{c} ± 0.64	2.59 ±0.16
200	26.35^{b} ± 0.64	27.13^{a} ± 0.68	2.96 ±0.15
Uninfected, ethanolic extract treated			[
10	23.75^{d} ± 0.61	25.28^{c} ± 0.65	6.44 ±0.52
100	25.38^{c} ± 0.63	26.30^{b} ±0.69	3.62 ±0.31
200	23.60^{d} ± 0.61	24.50^{c} ± 0.62	3.81 ±0.43
Infected, chloroquine treated			
5	22.60^{e} ± 0.60	23.63^{d} ± 0.61	4.56 ±0.46

Values are mean ± S. D n = 4. Mean values having different superscripts along the same column are significantly different (P<0.05).

Mean weights of mice organs

The organs of mice harvested for weight assessment were kidneys, livers, hearts and lungs. There was a significant decrease ($p<0.05$) in weight of kidneys and livers of infected untreated mice compared to the control (Table 10). However, there was no significant difference in weight of hearts and lungs of infected untreated mice compared to control.

The dose level of 10mg/kg body weight of aqueous extract in infected mice caused a significant decrease ($p<0.05$) in kidney and heart weight of the mice compared with the control. There was no significant difference ($p>0.05$) in weight of liver and lung of the infected mice treated with 10mg/kg body weight of the aqueous extract compared to the control. There was no significant difference ($p >0.05$) in the weight of kidney, heart and lung of the infected mice treated with 10mg/kg body weight of the aqueous extract compared with the infected mice treated with 100mg/kg body weight of the aqueous extract (Table 10). The dose level of 200mg/kg body weight of aqueous extract caused a significant decrease ($p<0.05$) in weight of kidney and liver organs of uninfected treated mice compared with the control.

Table 10. Mean weight(g) of mice organs after treatment with *L. lanceolata* aqueous leaf extract

Treatments Groups (mg / kg)	Organs in mice(g) Kidney	Liver	Heart	Lung
Negative control 10	$0.46^{a} \pm 0.08$	$1.41^{a} \pm 0.15$	$0.11^{b} \pm 0.04$	$0.15^{a} \pm 0.05$
Infected, untreated 0	$0.39^{b} \pm 0.07$	$1.04^{d} \pm 0.13$	$0.11^{b} \pm 0.04$	$0.14^{a} \pm 0.05$
Infected, aqueous extract treated 10	$0.35^{c} \pm 0.07$	$1.41^{a} \pm 0.15$	$0.13^{a} \pm 0.05$	$0.14^{a} \pm 0.05$
100	$0.34^{c} \pm 0.07$	$1.36^{b} \pm 0.15$	$0.13^{a} \pm 0.05$	$0.15^{a} \pm 0.05$
200	$0.41^{b} \pm 0.08$	$1.37^{b} \pm 0.15$	$0.11^{b} \pm 0.04$	$0.15^{a} \pm 0.05$
Uninfected, aqueous extract treated 10	$0.35^{c} \pm 0.07$	$1.42^{a} \pm 0.15$	$0.13^{a} \pm 0.05$	$0.13^{a} \pm 0.05$
100	$0.36^{c} \pm 0.07$	$1.20^{c} \pm 0.14$	$0.11^{b} \pm 0.04$	$0.14^{a} \pm 0.05$
200	$0.40^{b} \pm 0.08$	$1.29^{bc} \pm 0.14$	$0.11^{b} \pm 0.04$	$0.16^{a} \pm 0.05$
Infected, chloroquine treated 5	$0.27^{d} \pm 0.06$	$0.79^{e} \pm 0.11$	$0.06^{c} \pm 0.03$	$[0.14^{a} \pm 0.05$

Values are mean ± S. D n = 4. Mean values having different superscripts along the same column are significantly different ($P<0.05$).

The oral administration of ethanolic leaf extract of *L. lanceolata* in mice induced some changes in weight of the mice organs. The dose level of 100mg/kg body weight of ethanolic extract had a significant decrease ($p<0.05$) in weight of kidney, liver, and heart of infected mice compared with the control (Table 11). There was no significant difference ($p > 0.05$) in the weight of kidney, liver, and lung of the infected mice treated with 100mg/kg body weight of ethanolic extract compared with the infected mice at the dose level of 200mg/kg body weight of the ethanolic extract.

The dose level of 200mg/kg body weight of ethanolic extract in uninfected mice caused a significant decrease ($p < 0.05$) in liver and kidney of mice compared with the control. However, there was no significant difference in weight of heart, and lung of uninfected mice treated with 200mg/kg body weight of the ethanolic extract compared with the control.

Table 11. Mean weight (g) of mice organs after treatment with *L. lanceolata* ethanolic leaf extract

Treatments Groups (mg / kg)	Organs in mice(g) Kidney	Liver	Heart	Lung
Negative control 10	$0.46^{a} \pm 0.08$	$1.41^{a} \pm 0.15$	$0.11^{b} \pm 0.04$	$0.15^{a} \pm 0.05$
Infected, untreated 0	$0.39^{b} \pm 0.08$	$1.04^{d} \pm 0.12$	$0.11^{b} \pm 0.04$	$0.14^{a} \pm 0.05$
Infected, ethanolic extract treated			[	
10	$0.40^{b} \pm 0.08$	$1.29^{c} \pm 0.14$	$0.09^{c} \pm 0.04$	$0.15^{a} \pm 0.05$
100	$0.34^{c} \pm 0.07$	$1.32^{c} \pm 0.14$	$0.13^{a} \pm 0.05$	$0.15^{a} \pm 0.05$
200	$0.35^{c} \pm 0.07$	$1.32^{c} \pm 0.14$	$0.09^{c} \pm 0.04$	$0.15^{a} \pm 0.05$
Uninfected, ethanolic extract treated				
10	$0.37^{bc} \pm 0.08$	$1.36^{b} \pm 0.15$	$0.13^{a} \pm 0.05$	$0.14^{a} \pm 0.05$
100	$0.39^{b} \pm 0.08$	$1.37^{b} \pm 0.15$	$0.10^{b} \pm 0.04$	$0.16^{a} \pm 0.05$
200	$0.35^{c} \pm 0.07$	$1.37^{bc} \pm 0.15$	$0.1a^{b} \pm 0.05$	$0.14^{a} \pm 0.05$
Infected, chloroquine treated				
5	$0.27^{d} \pm 0.06$	$0.79^{e} \pm 0.11$	$0.06^{c} \pm 0.03$	$0.14^{a} \pm 0.05$

Values are mean $\pm$ S. D n = 4. Mean values having different superscripts along the same column are significantly different ($P<0.05$).

Discussion

The quality and quantity of phytochemicals extracted from the plant materials differed according to the solvent type used. Some reports show that ethanol extracts had more number and types of compounds in plant materials than other extraction solvents such as water [27]. The results of the investigation into the phytochemical compositions of aqueous and ethanolic leaf extracts of *L. lanceolata* employed in this study have revealed the presence of tannins, saponins, alkaloids, flavonoids, phenols, steroids, terpernoids, oxalate, and cardiac glycosides. The presence of these phytochemicals has also been reported in the leaves of *Anacardium occidentalis* [28]. The result is also in accordance with the reports of [29] whose phytochemical analysis of *Lophira lanceolata* revealed the presence of alkaloids, phenols, flavonoids, steroids, tannins and saponins. These constituents have been found in other plant products which exhibited antimalarial activity [30].

The result of the acute toxicity study indicated that none of the different doses (10 – 5000mg/kg) of aqueous and ethanolic leaf extracts in all the groups and phases caused mortality of mice for

over ten days up to 5000mg/kg body weight. The behavioural signs of toxicity observed in mice given 100mg/kg body weight and above included stretching, salivation, and reduced activity. These signs were not seen in 10mg/kg body weight dose group but progressed and became increasingly pronounced as the dose increased towards 5000mg/kg body weight. The median lethal dose (LD_{50}) was found to be ≥ 5000mg/kg body weight of the aqueous and ethanolic extract. The LD_{50} being greater than 5000mg/ kg body weight is thought to be safe as suggested by [20]. The results of acute toxicity study in this research work could explain the routine use of the plant by the local people for traditional management of malaria. Furthermore, the findings of this work are in agreement with the previous results of the acute toxicity study of the *Lophira lanceolata* leaf extracts on mice [31]. Also, these results are in conformity with the previous results of the acute toxicity study of the *Lophira lanceolata* leaf extracts on rats [32-33].

The oral administration of aqueous and ethanolic leaf extracts of *Lophira lanceolata* caused increases in the weights of the mice subjected to the experiment. The infected untreated mice had a decrease in mean body weight from 23.33g to 20.08g showing a 10.08% weight loss. The increase in weight observed in the treated mice showed that the animals responded to the treatment given.

This implies that the extract may act through mechanisms which are similar to those through which chloroquine acts [34]. Chloroquine is lethal to malaria parasites by causing the accumulation of toxic heme in the parasite food vacuole [35]. Heme may be toxic to the parasite by its interference with the nucleic acid biosynthesis [36-37]. The aqueous and ethanolic leaf extract of *Lophira lanceolata* showed significant dose-dependent antiplasmodial activity at the various concentrations. The dose level of 10mg/kg, 100mg/kg, 200mg/kg body weight of aqueous extract and the 5mg/kg chloroquine showed chemosuppressions of 12.09%, 20.60%, 60.44% and 89.56% respectively. Moreso, the dose level of 10mg/kg, 100mg/kg, 200mg/kg body weight of the ethanolic extract and 5mg/kg of the chloroquine drug showed chemosuppression of 12.64%, 17.31%, 41.21% and 89.56% respectively.

Conclusions

The study was undertaken to evaluate gametocyte dynamics and the effects of aqueous and ethanolic leaf extracts of *Lophira lanceolata* in mice infected with *Plasmodium bergei*. Thus, gametocytes resulting from sequestration of parasites seen on Day 0 should appear in circulation after Day 7. Although the level of gametocytaemia does not perfectly correlate with infectivity because it is easier to measure, it may be a useful indicator of an antimalarial's probable impact on malaria transmission. These results on gametocyte studies support previous studies demonstrating that the addition of artemisinins to standard antimalarial regimens reduces post- treatment gametocytaemia as gametocyte carriage did significantly decrease by the end of the 28 – day follow- up.

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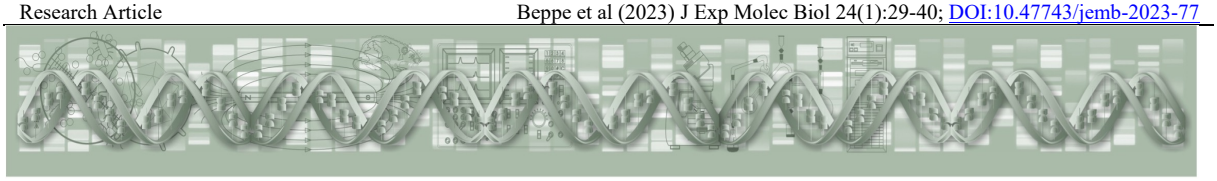

EVALUATION OF THE NEUROPROTECTIVE EFFECTS OF HYDROETHANOLIC EXTRACT OF *DIOSPYROS MESPILLIFORMIS* TRUNK BARK (EBENACEA) ON DIAZEPAM-INDUCED AMNESIA IN MICE

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Abstract

Diospyros mespilliformis (DM) is a plant of the Ebonaceae family that is commonly used in traditional medicine to treat epilepsy and schizophrenia. Radial arm mazes (RAM), Y mazes, and the novel object recognition test (NOR) were used to assess working and reference memory in mice. After the different tests, the hippocampus of the animals was separated for the determination of biochemical activities of markers such as acetylcholinesterase (AChE), malondialdehyde (MDA), and superoxide dismutase (SOD). The plant extract produced a significant rise in the percentage of alternation of mice in the Y-maze test, the time to observe the new object at the dose of 50 mg/kg ($p<0.01$) and the discrimination index ($p<0.001$) in all animals treated with the extract compared to the negative control. The result of the RAM test showed a significant decrease ($p<0.05$) in the number of errors on the reference memory in animals treated with the extract at 100 mg/kg. The extract significantly decreased AChE activity, and MDA concentration, and significantly increased SOD activity compared to the negative control group. These effects of the hydroethanolic extract on working and reference memories of DM extract would be related to the phenolic compounds quantified in the plant extract.

Keywords: *Diospyros mespilliformis*, amnesia, Diazepam, working memory, reference memory

Introduction

Amnesia is the pathological inability to learn new information or remember previously acquired information (Esther 2008). Amnesia is part of dementia, an estimated 46.8 million people worldwide suffered from dementia (Prince et al. 2013), among which 4 million were in Africa (ADI 2015). Many forms of brain damage can harm memory: physical aggression affecting the brain, diseases (brain tumors and infection), smoking (Ho et al. 2012), and medications such as benzodiazepines (Brault 2014). Diazepam's binding to GABA-ergic receptors results in chloride ion entry into the target cell and its hyperpolarization, which decreases postsynaptic excitability; thus, they have an inhibitory effect on signal transmission (Trincavelli et al. 2012). Benzodiazepines thus induce anterograde amnesia (Esther 2008). Diazepam induces alterations in brain structures with abnormal phosphorylation of the Tau protein which leads to the release of several free radicals (Ennaceur et al. 2009). However, the drugs used in the treatment of amnesia have several adverse effects and are unable to restore damaged structures because they cannot induce neurogenesis. Thus, research needs to promote the use of medicinal plants which

Received 20 December 2022; Accepted 21 February 2023; Published Online 23 February 2023; Published 3 April 2023

can be an alternative solution. Therefore, plants are an interesting source of new compounds in the search for bioactive molecules (Mohammedi 2013). This is the case of plants such as *Daniella oliveri* (Beppe et al. 2020), *Vigna subterranea* (Ngatanko et al. 2020), and *Ziziphus mucronata* (Foyet et al. 2019) whose therapeutic effects have been proven on neurological pathologies. *D. mespilliformis* belongs to the Ebenacea family and is used in traditional medicine for the treatment of epilepsy (Ali et al. 2021) and schizophrenia (Arbonnier 2008). The general objective of the present study was to evaluate the effects of hydroethanolic extract of *D. mespilliformis* trunk stem bark on diazepam-induced amnesia in mice.

Materials and Methods

Chemical substances: Diazepam, piracetam, and ketamine were obtained from Alfa-sigma (France); piracetam was dissolved in distilled water.

Plant material and extraction protocol

The bark of the trunk of *D. mespilliformis* was collected in Maroua, then authenticated at the School of Fauna of Garoua by comparison with a sample found there on the number HEFG/01404. Four thousand five hundred grams (4500 g) of fresh plant material was shade-dried for 16 days and then ground to powder. Then 500 g of the powder was macerated in 5 L of an ethanol/water mixture (80/20 v/v) for 72 h. The filtrate was concentrated with a rotary evaporator at 50°C and then oven-dried at 50°C. Forty-two grams (42 g) of crude extract was obtained after drying which lead to an extraction yield of 8.4%.

Animal matériel

Forty-two male mice between 9 and 10 weeks of age weighing between 25 and 30 g were used. These animals were purchased from the National Veterinary Laboratory (LANAVET) in Garoua.

Quantitative phytochemical analysis of the hydroethanolic extract of the trunk bark of *Dispyros mespilliformis*

Determination of total phenols: The determination of total polyphenols was performed according to the method of Folin-Ciocalteu (Mahmoudi et al. 2013).

Determination of flavonoids: Flavonoid was evaluated using the method of (Mimica-Duckic 1999).

Determination of tannins: The method by Bainbridge et al. (1996) was used to perform Tannins concentration

Determination of saponins: Saponins were performed using the method by Mohammedi (2013).

Animal material and experimental protocol

Forty-two mice (42 mice) were randomized into 6 groups of 7 animals each. They were treated for 14 days with the different solutions used in the experiment. However, diazepam. The animals were treated as follows: a normal control group received distilled water (DW; 10 mL/kg, p.o), a negative control group received distilled water (10 mL/kg, p.o) and diazepam (3 mg/kg, i.p) (DW + DZP), a positive control group received diazepam (3 mg/kg) and piracetam (200 mg/kg, p. o) (DZP + PIR), three test groups received diazepam (3 mg/kg) and subsequently, *D. mespilliformis* extract at different doses (50, 100 and 150 mg/kg; p.o) (DZP + 50 mg/kg, DZP + 100 mg/kg and DZP + 150 mg/kg). After all tests, the animals were sacrificed, and the hippocampi were removed for the determination of some biochemical parameters and histological studies.

Behavioral tests

Y-maze test: The Y-maze is used to assess the working memory of animals (Wolf et al. 2016). The alternation percentage is defined by the following relationship:

[(total number of alternations) / (total number of entries-2)] x 100.

Novel object recognition test: The novel object recognition test is a widely used test to access episodic-type memory (Sandrine 2018).
Radial arm maze test: The radial arm maze is an 8-armed device numbered 1 to 8 (48 cm x 12 cm) with a circular center of 32 cm, it is used to test working memory and spatial reference. At the end of four of the eight arms is a food reinforcer placed at least 50 cm from the center and out of sight of the animal (Hritcu and Nabeshima 2009).
Biochemical analysis of oxidative stress parameters
Hippocampal sampling: After behavioral testing, animals were anesthetized with ketamine (50 mg/ml) and diazepam (10 mg/2 ml). The brains were completely isolated and only the hippocampi were used for the preparation of the homogenate in triphosphate buffer (0.2 M; pH 7.4) kept cool at 4 °C.
Measurement of acetylcholine esterase (AChE) activity: The measurement of AChE activity was based on the Ellman method by spectrophotometry (Ellman 2007).
Determination of some parameters of oxidative stress
Determination of Malondialdehyde (MDA): The determination of the amount of MDA in the hippocampus was performed following the technique described by Wilbur et al. (1959).
Superoxide Dismutase (SOD) assay: The SOD assay was performed according to the principle described by Mishra 1972.
Realization of histological sections: Twenty-four hours (24 h) after behavioral experiments, mice were euthanized by decapitation under ketamine and diazepam anesthesia (10 mg/kg and 50 mg/kg B.W. i.p., respectively); hippocampi were harvested and fixed in 10% formalin for histopathological evaluation. Portions of the hippocampus were dehydrated, immersed in kerosene, deparaffinized, rehydrated, and stained with hematoxylin and eosin. The sectioned parts of the brain were then filmed, and the images were captured using a digital camera connected to an optical microscope (Scientifico, Haryana, India).
Statistical analysis
The results obtained were expressed as mean ± MSE. Data were analyzed by one-factor ANOVA (Y-maze) and two-factor ANOVA (NOR and RAM) followed by Dunnett's and Bonferroni's post-tests, respectively. All analyses were performed using Graph Pad Prism version 8.0.1 for Windows. Results were considered significant for $p<0.05$.

Results and discussion

Quantitative phytochemical test of the hydroethanolic extract of *Diospyros mespilliformis* trunk bark

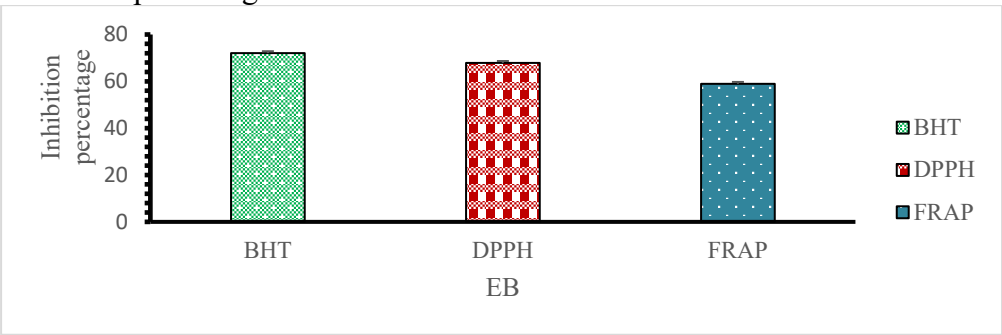
The phytochemical screening performed on the hydroethanolic extract of *D. mespilliformis* trunk bark showed that flavonoids are the most abundant polyphenols with an amount of 55.119 equivalent (eq) of quercetin/100 g of dry extract followed by saponins with an amount of 38.803 equivalent (eq) of galactose/100 g of dry extract (Table 1). Tannins were the least abundant polyphenols in the extract with an amount of 5.239 equivalent (eq) of catechin/100 g dry extract.

Table 1. Results of quantitative phytochemical screening of selected secondary metabolites of the hydroethanolic extract of *Diospyros mespilliformis* trunk bark.

Secondary metabolite class	Secondary metabolite concentration
Total polyphenols	73.146 mg Eq gallic acid/100 g ES
Flavonoids	55,119 mg Eq quercetin/100 g ES
Tannins	5,239 mg Eq catechin/100 g ES
Saponins	38,803 Eq galactose/100 g ES

Eq = equivalence, ES = dry extract

In vitro antioxidant activity: The antioxidant potential of the hydroethanolic extract of *Diospyros mespilliformis* trunk bark was measured using the DPPH and FRAP tests (Fig. 1). The results of these two tests revealed that the extract has a DPPH inhibition percentage of 67.87% and FRAP of 58.94% in comparison to the positive control (BHT) which has an inhibition percentage of 72.05%.



BHT: positive; DPPH: inhibition of DPPH extract; FRAP: inhibition of FRAP extract; Each histogram represents the mean ± MSE.

Effect of hydroethanol extract of *Diospyros mespilliformis* trunk bark on short-term memory

Figure 2 shows the percentage of alternation of animals. Animals treated only with diazepam showed a significant decrease ($p < 0.001$) in the percentage of alternations (56.5 ± 2.02%) compared to animals in the normal control group (90.0 ± 0.49%). The hydroethanolic extract of *D. mespilliformis* bark significantly ($p < 0.001$) increased the percentage of alternation at all doses (92.70 ± 1.86 %, 84.7 ± 2.91%, and 74.00 ± 1.04 % at 50, 100, and 150 mg/kg, respectively) compared to the negative control group. The reference substance piracetam significantly ($p<0.01$) increased the percentage of alternation of the positive control (68.9±0.84%) compared to the negative control.

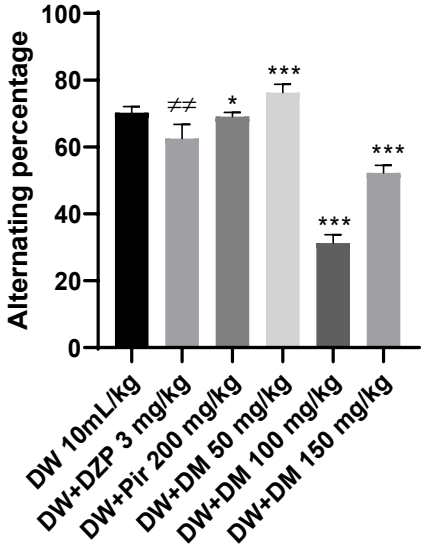


Figure 2. Effect of hydro-ethanol extract of *Diospyros mespilliformis* barks on the percentage of alternation of the Y-Maze test after 14 days of treatments. Each histogram represents the mean ± MSE, DZP = diazepam for negative control; DW= distilled water; Pir = piracetam for positive control; DM= the hydro-ethanolic extract of *Diospyros mespilliformis*; $^{###}P < 0.001$ significant difference of the negative compared to the normal control; $^{***}P < 0.001$, $^{**}P < 0.01$ significant difference compared to the negative control

Effect of hydroethanolic extract of *D. mespilliformis* trunk bark on exploration time and discrimination index in the novel object recognition test

The results in Figure 3 below show the exploration time (Figure 3A) and discrimination index (Figure 3B) of animals. Animals in the negative control group showed a significant decrease in novel object exploration time ($p<0.001$) and discrimination index ($p<0.01$) compared to animals in the normal control group. On the other hand, the hydroethanolic extract of *D. mespilliformis* induced in the animals a significant increase ($p<0.01$) in the exploration time of the novel object at the dose of 50 mg/kg as well as a significant increase ($p<0.001$) in the discrimination index at all doses tested, in comparison with the negative control. Piracetam induced a significant increase ($p<0.001$) in the time to explore the novel object and in the discrimination index of the animals compared to the negative control.

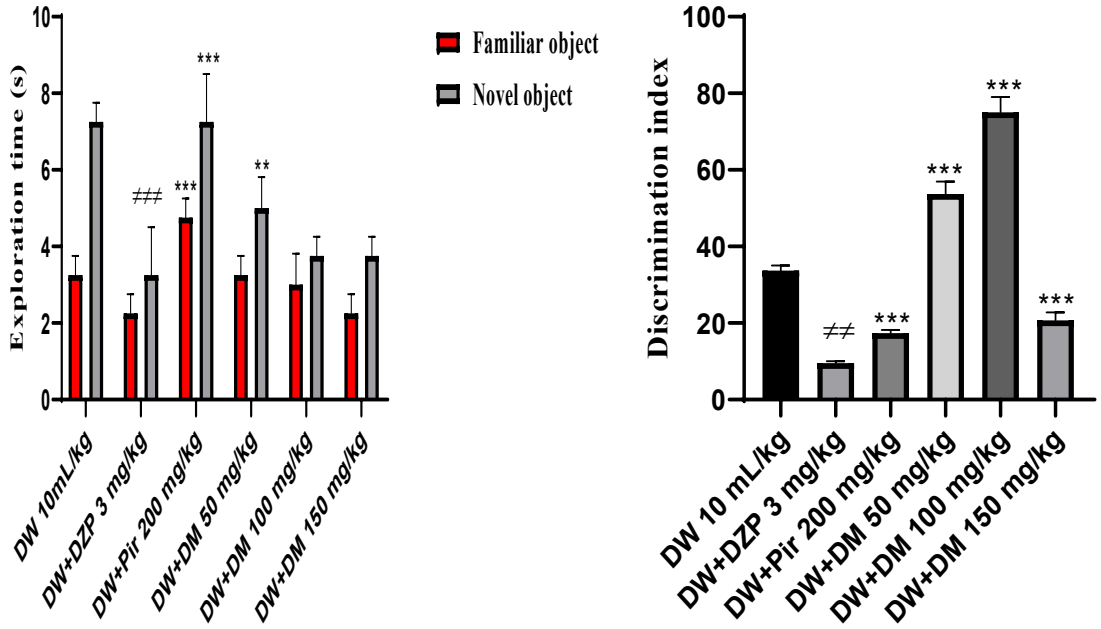


Figure 3. Effect of hydroethanol extract of *Diospyros mespilliformis* trunk bark on scanning time (A) and discrimination index (B). Each histogram represents the mean ± MSE. (n=5), DZP = diazepam; DW= distilled water; Pir = piracetam positive control; DM= the hydroethanolic extract of *Diospyros mespilliformis*; $^{\#\#}P < 0.01$ significant difference of the negative control compared to the normal control; $^{***}P < 0.001$ a significant difference of the test batches compared to the negative control

Effect of the hydroethanol extract of *Diospyros mespilliformis* barks on baseline memory in the 8-arm maze test

Figure 4 below shows the effect of the extract on the baseline memory. These results show that diazepam induced a significant increase ($p<0.05$ on days 5 and 6) and ($p<0.001$ on days 7) in the number of errors in reference memory of animals in the negative control group compared to animals in the normal control group. The hydroethanolic extract of *D. mespilliformis* barks at the dose of 50 mg/kg significantly ($p<0.05$) reversed the number of errors (4.00, 2.75, 2.00, and 1.25) on the reference memory of the animals in comparison to the animals of the negative control group (5.75, 5.75, 5.75, and 5.00) at days 4; 5; 6 and 7 respectively. Similarly, the 100 mg/kg dose extract significantly reversed the number of errors (2.25 and 2.50) in baseline memory on days 6 and 7. Piracetam, the reference product, induced a significant decrease ($p<0.05$) in the number of errors (2.00) in the reference memory of animals in the positive control group compared to the negative control on day 7.

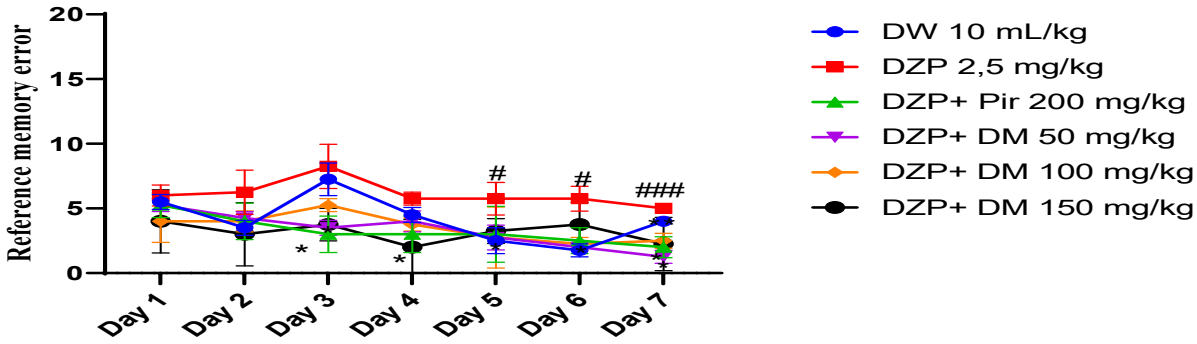


Figure 4. Effect of hydroethanol extract of *Diospyros mespilliformis* trunk bark on baseline memory. Each point represents the mean ± MSE. DZP = diazepam negative control; DW= distilled water normal control; Pir = piracetam positive control; DM= the hydroethanolic extract of *Diospyros mespilliformis*; $^{###}P < 0.001$ significant difference of the negative control compared to the normal control; $^{*}P < 0.05$ significant difference of the test and positive batches compared to the negative control

Effect of hydroethanolic extract of *Diospyros mespilliformis* bark on acetylcholine esterase activity

Figure 5 represents the activity of AchE in the hippocampus of mice. It appears that AchE activity was significantly ($p<0.001$ and $p<0.05$) decreased in animals treated with the hydroethanolic extract of *D. mespilliformis* bark at doses of 50 mg/kg and 100 mg/kg, respectively, compared to the negative control. The reference substance piracetam significantly ($p<0.001$) decreased the percentage of alternation in the positive control compared to the negative control.

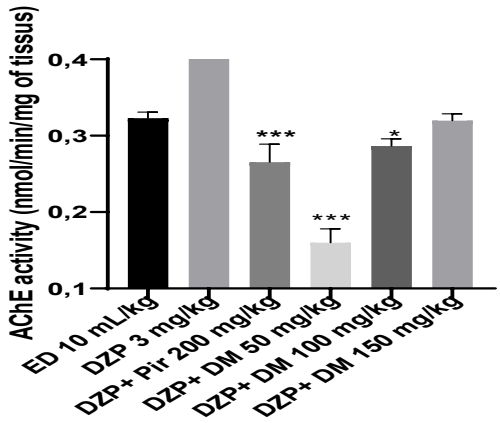


Figure 5. Effect of hydroethanol extract of *Diospyros mespilliformis* bark on acetylcholine esterase activity. Each bar represents the mean ± MSE. *P < 0.05 a significant difference from the negative control. AchE= acetylcholine esterase; DZP = diazepam for negative control; DW= distilled water; Pir = piracetam for positive control; DM= the hydro ethanolic extract of *Diospyros mespilliformis*.

Effect of hydroethanol extract of *Diospyros mespilliformis* barks on the concentration of Malondialdehyde (MDA), superoxide dismutase (SOD) activity

Animals in the negative control group showed a significant increase ($p<0.05$) in MDA concentration compared to animals in the normal control group after 14 days of treatment (Figure 6A). The hydroethanolic extract of *D. mespilliformis* bark at the dose of 50 mg/kg, 100

mg/kg, and 150 mg/kg significantly ($p<0.001$) decreased the concentration of MDA and significantly ($p<0.001$) increased the SOD activity of the animals compared to the animals in the negative control group (Fig. 6B). The reference substance piracetam significantly ($p<0.05$) decreased the MDA concentration and significantly ($p<0.001$) increased the SOD activity of the animals compared to the negative control.

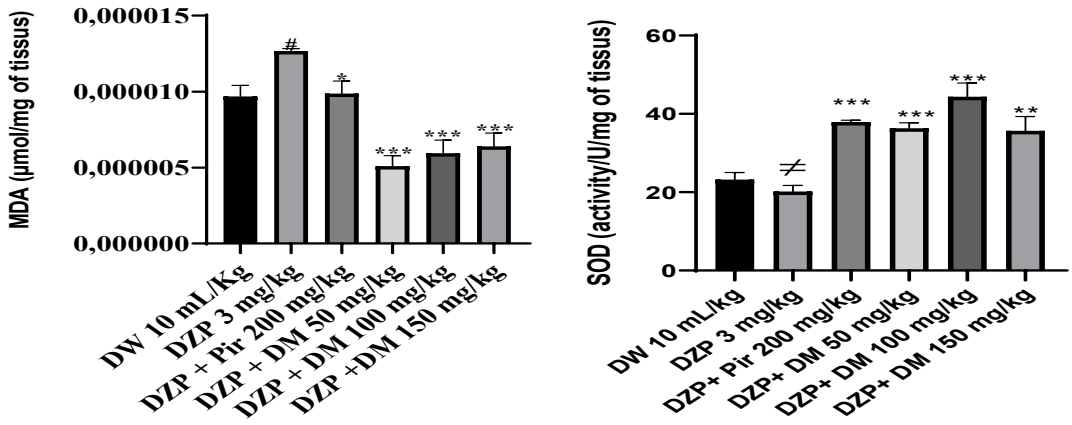


Figure 6. Effect of hydroethanolic extract of *Diospyros mespilliformis* trunk barks on MDA concentration (A) and SOD activity (B) in mouse hippocampus. Each histogram represents the mean ± MSE. DZP = diazepam for negative control; DW= distilled water for normal; Pir = piracetam for positive control; DM= the hydro ethanolic extract of *Diospyros mespilliformis;* $^{\#}P < 0.05$ significant difference of the negative compared to the normal control; $^{***}P < 0.001$ and $^{*}P < 0.05$ a significant difference of the test and positive batches respectively compared to the negative control

Effect of hydroethanolic extract of *Diospyros mespilliformis* trunk bark on hippocampal sections

Histological analysis (Figure 7) shows in the normal control a normal architecture of the hippocampus, with neurons of intact appearance in the different layers (GD, CA1, CA2, and CA3). In the negative control animals that received diazepam, a pathological modification (neuronal vacuolization) was observed in the different hippocampal layers. The animal groups that received the plant extract (at different concentrations) and piracetam showed a microstructure of the hippocampus close to that of the normal control.

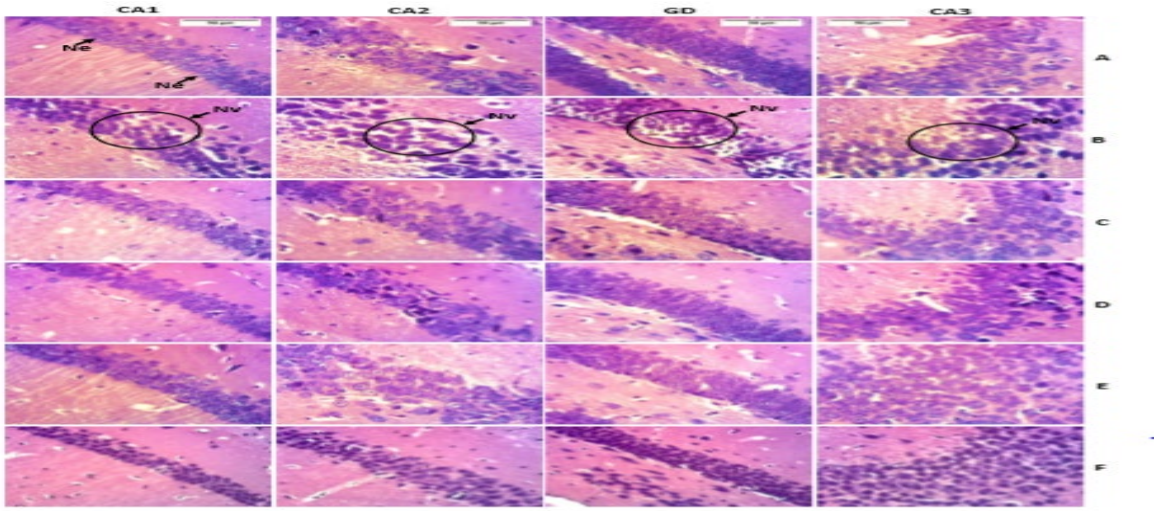


Figure 7. Microphotographs of the dentate gyrus (x250) and Ammon horns 1, 2, and 3 (X250) of the hippocampus; Hematoxylin-eosin staining. CA1, 2, 3 = Ammon corn 1, 2 and 3; GD = Dentate gyrus; Ne = Neuron; Vn = Vacuolated neuron; A = Normal control; B = Negative control; C = Positive control; D, E, F = Test batches receiving the extract at doses of 50, 100 and 150 mg/kg respectively

The aqueous extract of the plant was used to assess the cognitive aspect of memory through behavioral tests such as the Y-maze, radial arm maze, and novel object recognition test. Subsequently, biochemical analyses were performed to verify the observations of the behavioral studies. The Y-maze is used to measure the impact of a substance on short-term memory (Tolman 1924). An increase in the percentage of alternations reflects an improvement in short-term memory (Krishna et al. 2016). Treatment with the plant extract at all doses resulted in a significant increase in this percentage, compared to the negative control. Diazepam reduced the memory capacity of the negative control animals by binding to GABA-ergic type A receptors, resulting in a massive entry of chlorine ions into the target cell, leading to hyperpolarization and a decrease in neuronal excitability (Trincavelli et al. 2012). Further tests such as the arm maze test are needed to confirm these observed effects. This test measures the learning abilities in the environment where the animal is placed, as well as its short and long-term memory abilities (Beppe et al. 2014). The number of working memory errors in this test did not show any significance between the groups of animals. This could be because these animals were able to retain residual memory. Cohen and Squire (1980) showed that in the case of severe memory loss, short-term memory is preserved. Pretreatment with DM hydroethanolic extract significantly reduced the number of errors in baseline memory in mice. Beppe et al (2014) showed that the decrease in the number of errors on reference memory could be related to memory improvement in parkinsonian rats. The novel object recognition test is regularly used to demonstrate episodic-type memory (Sandrine 2018). Diazepam-treated animals (3 mg/kg) showed a significant decrease in novel object viewing time and discrimination index. Diazepam could have caused alterations in brain structures leading to memory impairment in this negative control group, as reported by Ennaceur et al (2009). Indeed, novelty recognition is based on the natural preference for novelty displayed by rodents to assess cognitive alterations in animal models of neurodegenerative disorders (Ennaceur et al. 2009). Treatment of animals with DM extract at all doses showed an increase in the discrimination index compared to the negative control. Acetylcholine is one of the most important neuromodulators in the cerebral cortex (Jaffard 1994). It is importantly and involved in the neural circuits of working memory, attention, episodic memory and spatial memory (Brault 2014). This

neuromodulator is degraded at the synaptic cleft by AChE to choline and acetate (Sanson 2009). Benzodiazepines such as diazepam primarily stimulate GABA receptors, and thus hyperpolarise and reduce serotonin and acetylcholine levels in the brain. A significant decrease in AChE enzyme activity was noted in animals treated with DM extract at 50 mg/kg and 100 mg/kg. The decrease in acetylcholine esterase activity could improve the cholinergic transmission process and therefore also improve learning and memory (Kouémou et al. 2017, Kim et al. 2018). Stress implies in the process of brain damage, as it causes direct denaturations of the blood-brain barrier (BBB) junction proteins (Elizabeth 2010). Diazepam induces alterations in brain structures with abnormal phosphorylation of the Tau protein resulting in the release of several free radicals (Ennaceur et al. 2009). MDA is derived from lipid peroxidation which leads to an increase in the permeability of the blood-brain barrier in vitro (Mertsch et al. 2001) and thus, hypermethylation of the promoter region of the adhesion protein E-cadherin, resulting in its loss of expression, as well as an alteration of the BBB function (Lim et al. 2008). Concerning protein damage, it has been suggested that reactive species can modulate calcium channel function and thus mediate dysfunction via Ca^{2+} cytotoxicity (Brown and Davis 2002). Animals treated with DM hydroethanolic extract at all doses showed a significant reduction in MDA concentration compared to the negative control. Antioxidants are substances capable of neutralizing or reducing free radical damage in the body while allowing the maintenance of non-cytotoxic concentrations of ROS (reactive oxygen species) at the cellular level (Mohammedi, 2013). The flavonoids and tannins in this DM extract could have acted to decrease the concentration of MDA. The extract might possess the ability to inhibit the transformation of hydrogen peroxide to hydroxyl radical by reducing ferric iron (Fe^{3+}), which is essential for the formation of hydroxyl radical to ferrous iron (Fe^{2+}) in vitro. This reducing power could be due to the presence in the extract, of phenolic compounds including flavonoids and hydrolyzable tannins (Alioune et al. 2015). The presence of the hydroxyl group on the chemical structure of flavonoids and tannins allows them to trap radical species by giving up an electron (Boubekri 2014). Once their electrons are surrendered, they become radicals but remain stable due to their aromatic system which allows them to capture other radical species (Dai and Mumper 2010). All the above statements taken together further confirm the antioxidant potential of the plant extract evaluated in this study. Mohammedi (2013) showed that flavonoids are antioxidants capable of scavenging free radicals and that tannins contain significant antioxidant properties that act as scavengers and proton donors against lipid free radicals produced during lipid peroxidation; yet MDA is a product of lipid peroxidation. SOD is the first line of antioxidant defense (Sfar et al. 2013). The assay from homogenates of animals treated with the extract at all doses showed a significant increase in SOD activity compared to the negative control.

In this work, DZP caused alterations in different structures of the hippocampus. Pretreatment with *D. mespilliformis* hydroethanolic extract protected the hippocampus from the neurotoxic effects of DZP. Flavonoids protect vulnerable neurons, improving existing neuronal functions and stimulating neuronal regeneration (Vauzour et al. 2010).

Conclusions

This work was undertaken to evaluate the impact of the hydroethanolic extract of *Diospyros mespilliformis* bark on diazepam-induced amnesia in mice. The hydroethanolic extract of DM trunk bark improved the memory of amnesic mice by decreasing MDA levels, increasing SOD activity and decreasing acetylcholine esterase levels in the hippocampus of animals. The antioxidant properties of this extract are thought to be due to the secondary metabolites it contains, which mainly protect the hippocampus against the neurotoxic effects of diazepam.

Abbreviatons: CA: Cornu Ammonis; DZP : diazepam; *D. mespilliformis*: *Diospyros mespilliformis*; BW: Body weight; YM: Y-maze; AChE: Acetylcholine esterase; MDA: Malondialdehyde; SOD: Superoxide dismutase.

Acknowledgments
The authors are very grateful to the Head of the Department of Biological Sciences, Faculty of Science, University of Maroua, Cameroon, and the Head of the Animal Physiology Laboratory, Department of Biology, and Animal Physiology, Faculty of Science, University of Yaoundé I, Cameroon for providing the facilities for this work.

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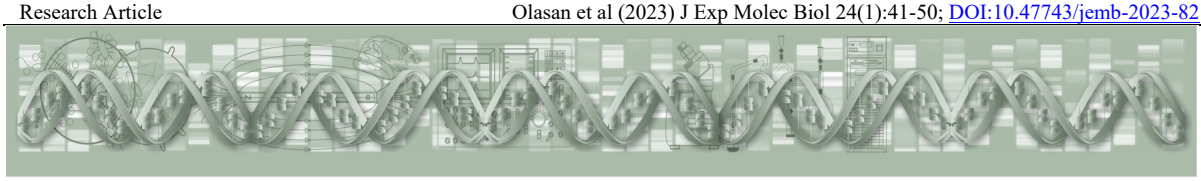

GENETIC DIVERSITY AND PHYLOGENETICS OF FOUR RELEASED COWPEA (*VIGNA UNGUICULATA* (L.) WALP) VARIETIES (FUAMPEA-1, FUAMPEA-2, FUAMPEA-3 AND FUAMPEA-4) USING SIMPLE SEQUENCE REPEATS MARKERS

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Abstract

Cowpea improvement has continued to play key roles in the quest for national food security. This work elucidated the molecular genetic diversity and phylogenetics among four released cowpea varieties (FUAMPEA-1, FUAMPEA-2, FUAMPEA-3 AND FUAMPEA-4). DNA was extracted from 14 day old seedlings using the CTAB method. A total of nine (9) SSR (Simple Sequence Repeats) markers were employed in DNA amplifications. Binary matrix was generated from banding profiles of gel images and uploaded into the Minitab 17.0 software for analysis. Polymorphic Information Content (PIC) was computed for the primers and cowpea varieties. Cluster analysis was carried out using the Single Linkage method measured on Euclidean Distance. Four primers (CLM1190, CLM0342, RB38 and RB7) produced maximum of two bands each resulting in 15.4% polymorphism and 0.977 Polymorphism Information Content (PIC). Varietal Polymorphic Content (VPC) varied from 15.4% in FUAMPEA-2 as the lowest to 38.5% in FUAMPEA-1 as the highest. FUAMPEA-1 had the lowest genetic similarity index (19.82). Dendrogram further showed that FUAMPEA-2 and FUAMPEA-3 were more closely related than others with about 46.55 similarity index. Therefore, FUAMPEA-1 was the most divergent and genetically diverse variety followed by FUAMPEA-4. These are potential genetic resource material that can used to develop more cultivars when their inherent agronomic traits and genes coding for the traits are identified and tapped.
Keywords: Cowpea, Breeding, Phylogenetics, Molecular markers, Food security

Introduction

Cowpea (*Vigna unguiculata* (L.) Walp), family Fabaceae, is an important leguminous food crop of the sub-Saharan African countries and it has become a staple food produced and consumed by almost every home in Nigeria. The grain is a source of cost effective plant based dietary protein (25%) and amino acids in addition to high fiber, vitamin and mineral contents needed to address malnutrition and poverty in developing countries (Aboki et al. 2013, Ahenkora et al. 2018). About 83.4% of the world's overall production comes from Africa while over 80% of African production comes from West Africa including Nigeria being the largest producer of

Received 28 December 2022; Accepted 6 March 2023; Published Online 16 March 2023; Published 3 April 2023

cowpea in Africa (Omoigui et al. 2016). The crop is well adapted to drought. It competes favourably well with other plants while playing an important role in nitrogen fixation, a process that adds nutrients to the soil **(**Uzogara and Ofuya 2012, Taiwo et al. 2014**)**. Although there have been successes of cowpea production in the country, the crop is challenged by numerous biotic and abiotic stresses that limit its productivity or consumption (Omoigui et al. 2017). There is need for continuous improvement to ensure its sustainable production to feed the growing human population. The use of modern biotechnology cannot be over-emphasized in this regard (Singh et al. 2012).

To solve the numerous constraints to cowpea production, plant breeders have initiated many improvement programmes aimed at addressing specific challenges to meet the demand of farmers and consumers ((Singh et al. 2012). There are varieties that have been improved for resistance against parasitic weeds, fungal and bacterial diseases (Omoigui et al. 2016, 2017). Some varieties have been improved for nitrogen efficiency use and tolerance to pesticides and drought (Boukar and Fatokun 2019). The lack of genetic diversity has made further improvement in cowpea varieties an arduous task. New variation is critical for introducing interesting characteristics into breeding programs. Diversity in plant genetic resources is needed to provide opportunities for plant breeders to develop new improved cultivars with desirable traits. Varietal development is a crucial aspect of crop improvement that provides genetic resource to improve local landraces to achieve national food security (Boukar and Fatokun 2019).

Federal University of Agriculture Makurdi Nigeria (FUAM) in collaboration with IITA (International Institute of Tropical Agriculture) has been in the front burner of cowpea improvement programmes mainly to improve yield, maturity time and resistance to parasitic weeds, pests and diseases (Omoigui et al. 2015, 2016, 2017). Consequently, the National Variety Release Committee (NVRC) approved the release of two improved varieties (FUAMPEA-1 and FUAMPEA-2) in 2016 and additional two varieties (FUAMPEA-3 and FUAMPEA-4) in 2022. The overall aim is to boost cowpea production to achieve food security not only in Nigeria but also in Africa in addition to boosting farmers' income and improving their livelihood. Studies on the genetic diversity and phylogenetic relationship have not been conducted on these new varieties. This aspect is considered important in plant breeding and improvement programmes (Boukar and Fatokun 2019, Olasan et al. 2020). Simple Sequence Repeats (SSR) markers have been applied on a number of crops for cultivar identity, genetic diversity, determination of evolutionary relationship, marker assisted selection and construction of linkage maps (Dughdugh et al. 2017, Olasan et al. 2020, Tersoo et al. 2021, Aguoru et al. 2022). These repetitive DNA regions are reported to be polymorphic co-dominant markers with high resolutions (Olasan et al. 2020). The aim of this work was to assess the genetic diversity and phylogenetics among four newly released cowpea varieties using specific SSR markers.

Materials and Methods

Study area and plant sample collection

This study was carried out in the Molecular Biology Laboratory of the Federal University of Agriculture Makurdi (Now Joseph Sarwuan Tarka University Makurdi). Seeds of four new cowpea varieties developed by the Federal University of Agriculture Makurd (FUAM) were sourced from the Seed Store of the institution. They were: FUAMPEA-1, FUAMPEA-2, FUAMPEA-3 and FUAMPEA-4.

Molecular markers

A total of nine (9) cowpea specific SSR (Simple Sequence Repeats) primers were employed in DNA amplifications. They were part of the routine primers used in cowpea breeding work. The names and sequences of the primers are given in table 1.

Seed planting for molecular studies

Four seeds of each variety were planted in separate pots filled with soil inside the Screen House of the Department of Plant Breeding and Science of the same institution. Young leaves were collected from 14 day old seedlings.

DNA extraction

CTAB (Cetyltrimethylammonium bromide) method of DNA extraction method was used (Tersoo et al. 2021). Two leaves were placed in silica gel for three days to dry. The crispy dry leaves were squeezed into 2ml eppendorf tubes containing two steel balls and grounded vigorously using a vortex for one minute. Buffer (1M Tris-Hcl, 0.5M EDTA, 5M NaCl, 2-Mecarptoethanol) was added and incubated in a water bath for 30minutes at 60°C. Exactly 600uL chloroform: Isoamylalcohol (24:1) was added and spun for 10minutes at 4000rpm. The upper layer was transferred into new tubes and this step was repeated. 600uL of ice cold 2-propanol was added into the supernatant and inverted for few seconds. Tubes were kept in -20°C freezer to precipitate, after precipitation tubes were then centrifuge for 35minutes at 4000rpm to form pellets at the bottom of the tube and then supernatant was decanted. The pellets were washed with 400uL of 70% ethanol, centrifuge for 15minutes and decant ethanol. This process was repeated and the pellets were allowed to air dry for few hours by placing the opened tubes on paper towel. The dried pellets were suspended in 90uL of molecular grade water.

Polymerase chain reaction

SSR based PCR protocol was used (Omoigui et al. 2015). Each reaction contained 25uL of double distilled water and 1.5 µl of one specific primer pair mixed in a PCR tube already containing a customized BIONEER® Accupower PCR Premix (Top DNA polymerase (1U), dNTPs (250µM), Tris-HCl of 9.0 pH (10mM), KCl (30mM), $MgCl_2$ (1.5mM), stabilizer and tracking dye). The PCR tubes were loaded on the thermal cycler and programmed with the following temperature gradient profile as follows: 94°C denaturation temperature, 55°C annealing temperature, 72°C elongation temperature and 35cycle running for 3hours on a thermal cycler (Applied Biosystem in Life Technology version 2720).

Agarose gel electrophoresis

Exactly 0.8% agarose powder was poured into a beaker containing 1xTAE buffer and was swirled gently. The beaker was placed into the microwave for few minutes till the solution became crystal. The solution was allowed to cool and 5uL of EtBr was added and poured on an already gel plate with comb. Solution was placed into the electrophoresis tank (Galileo Bioscience tank connected to Consort EV243 electrophoresis power supply). The comb was removed gently 5uL of DNA sample was added into the PCR tube while 2uL of 6x loading dye was also added and spun for few seconds. Sample was gently loaded into the wells using pipette and finally 5uL of ladder was loaded. The gel ran at 120v for 45 minutes. DNA purity and quality was checked using UV spectrometer light (Omoigui et al. 2015).

DNA band visualization

The banding pattern of the samples resolved on agarose gel was viewed on a UV Bench top trans-illuminator and the gel image was captured using a camera for band scoring. Only distinct bands were scored as present (1) or absent (0) (Omoigui et al. 2015).

Data analysis

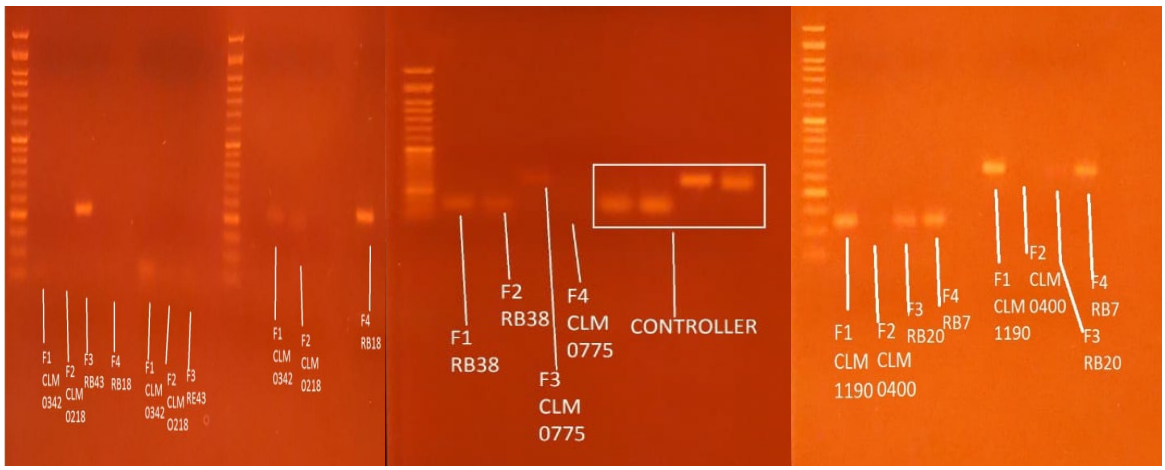
Binary matrix was generated from DNA banding profiles of gel images and uploaded into the Minitab 17.0 software for analysis. Polymorphic bands and % polymorphism were calculated for each SSR primer used. Polymorphism Information Content (PIC) was calculated using the formula adopted by Weir (1990) and Tersoo et al. (2021).

$PIC = 1 - \Sigma pi2$

Where *pi* is the frequency of the *i* th allele for each SSR marker *i* th summed across all alleles for the loci. Varietal Polymorphic Content (VPC) was calculated and expressed as percentages. Cluster analysis was carried out in two forms (i) the markers and (ii) the varieties. This was done using the Single Linkage method measured on Euclidean Distance resulting in the dendrogram (Olasan et al. 2018).

Results and discussion

Selected gel images produced from the study are shown in Plates 1a-c while banding patterns are pictorially given in figure 1. All the primers gave good amplification of DNA of the four varieties of cowpea studied. The resulting number of band depends on the SSR primer type and the cowpea variety. Three primers were clearly resolved in FUAMPEA-1 (CLM0342, CLM1190 and RB38) and FUAMPEA-3 (CLM0775, RB20 and RB43) while two primers were resolved in FUAMPEA-2 (CLM0218 and RB38) and FUAMPEA-4 (RB18 and RB7). Therefore, two to three primers have helped to differentiate the released varieties. This study is in agreement with previous reports on the high resolution of SSR markers in differentiating heterozygosity of traits as co-dominant markers (Jonah et al. 2011, Mason 2015, Tersoo et al. 2021). Different alleles may exist at a given SSR locus and this makes SSR markers more useful than other molecular markers including single nucleotide polymorphism (SNP) marker because repetitive regions linked to traits are highly unstable due to their high rate of mutation (Mason, 2015).



Plates 1. Selected Gel images of DNA from cowpea varieties as amplified by nine SRR primers

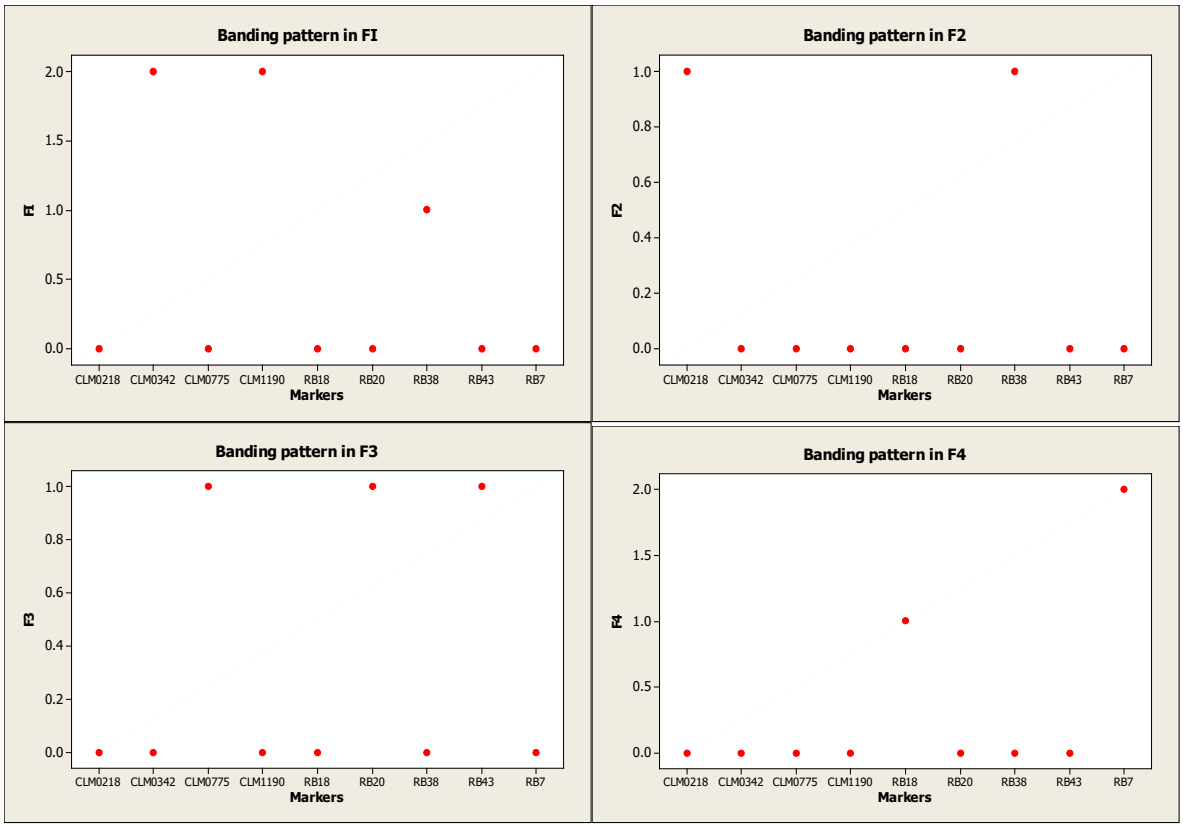


Figure 1. DNA Banding pattern of SSR molecular markers in the four varieties of cowpea (F1-F4 respectively)
Legend: FI= FUAMPEA-1; F2= FUAMPEA-2; F3= FUAMPEA-3; F4= FUAMPEA-4

Some levels of clustering were found among the primers based on their relatedness (Figure 2) as observed between CLM1190 and CLM0342 as well as CLM0775, RB43 and RB20. The divergent primers were RB38, CLM0218, RB7 and RB18. Four primers produced two bands each (CLM1190, CLM0342, RB38 and RB7) resulting in 15.4% polymorphism and 0.98 Polymorphic Information Content (PIC). Other primers produced one band each with 7.7% polymorphism and 0.941 PIC. All the primers had average of 1.4 polymorphic bands, 11.1% polymorphism and 0.957 PIC (Table 1). All the primers used in this study are highly informative as revealed by their high PIC values. According to Nelson *et al*. (2016), PIC values of 0.5, 0.4 and 0.2 are classified as highly informative, moderately informative and little informative respectively. The average PIC of 0.957 reported in this study was higher than values obtained in most breeding reports on SSR markers (Dhaliwal et al. 2014, Kumar et al. 2016, Dughdugh et al. 2017, Alfred et al. 2019, Tersoo et al. 2021). A marker is said to be polymorphic if it has at least two alleles and the allele frequency is up to 0.9 (Mason 2015). Therefore, four markers including CLM1190, CLM0342, RB38 and RB7 may be selected for cowpea improvement programmes because of their higher percentage polymorphism, PIC values and existence of heterozygosity for different traits. Moreover, other regions of the DNA identified by these primers may be coding for unique traits of breeding values. This outcome is consistent with the report given by Omoigui et al. (2015) who determined the suitability and use of two molecular markers to track race-specific resistance to *Striga gesnerioides* in cowpea.

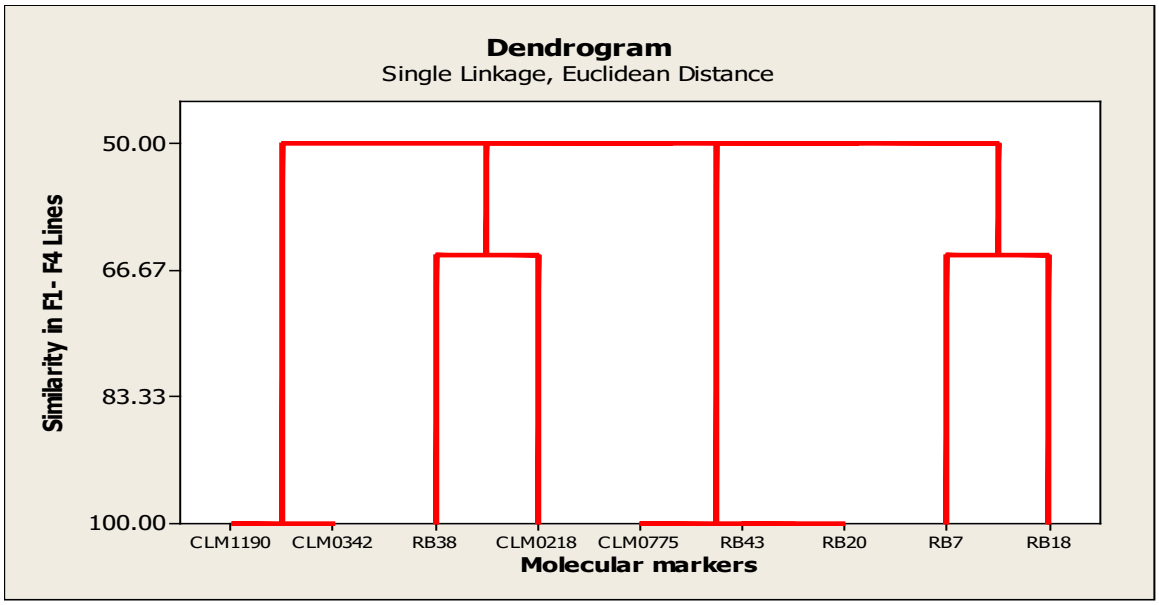


Figure 2. Relationship in banding pattern of SSR primers

Table 1. Primer name, Sequence and Polymorphism Information Content (PIC)

Primer name	Sequence	∑Polymorphic bands	% Polymorphism	PIC 1 - Σ *pi*2
CLM1190	ATTTGGCTGAATTGTTTCCA	2	15.4	0.9775
CLM0342	GGATTGGATATGTGTCTGGC	2	15.4	0.9775
RB38	GCGGCCGCTGCTCGTTCCCG	2	15.4	0.9775
CLM0218	TTTCCGATTTGCGATTTTTA	1	7.7	0.9410
CLM0775	GTGGCAGCACAAGTTAGTAG	1	7.7	0.9410
RB43	CCATGGTCGCCCCTGCTGCACCTTG	1	7.7	0.9410
RB20	CCATGGGGGCATCAACCTTGG	1	7.7	0.9410
RB7	GGGCGTTAATTAAGCCCACACA	2	15.4	0.9775
RB18	AAGCTTACTTGTACAGCTCGTCCATGCCG	1	7.7	0.9410
Total		13	100	8.615
Mean		1.4	11.1	0.957

Varietal Polymorphic Content (VPC) varied from 15.4% in FUAMPEA-2 as the lowest to 38.5% in FUAMPEA-1as the highest. FUAMPEA-3 and FUAMPEA-4 had VPC value of 23.1% each slightly below the mean VPC of 25.0% (Figure 3). Based on the phylogenetic relationship among the varieties, FUAMPEA-1 was the most divergent variety with 19.82 similarity index followed by FUAMPEA-4. Dendrogram further showed that FUAMPEA-2 and FUAMPEA-3 were more closely related than others with about 46.55 similarity index (Figure 4). This phylogenetic construction based on genetic diversity is useful in breeding

programme to make an informed decision about a potential genetic material with high breeding value whose genes could be tapped. It has given us an understanding of the genetic changes that must have taken place in the course of the breeding work. This is consistent with findings in other crops (Aguoru et al. 2015, 2022). As new varieties of cowpea are developed and released, plant breeders need to utilize them to improve other varieties to achieve national food security. This position is fully supported by plant breeders (Xu et al. 2010, Viswanatha et al. 2011, Atnafua and Endashaw 2014; Dughdugh et al. 2017).

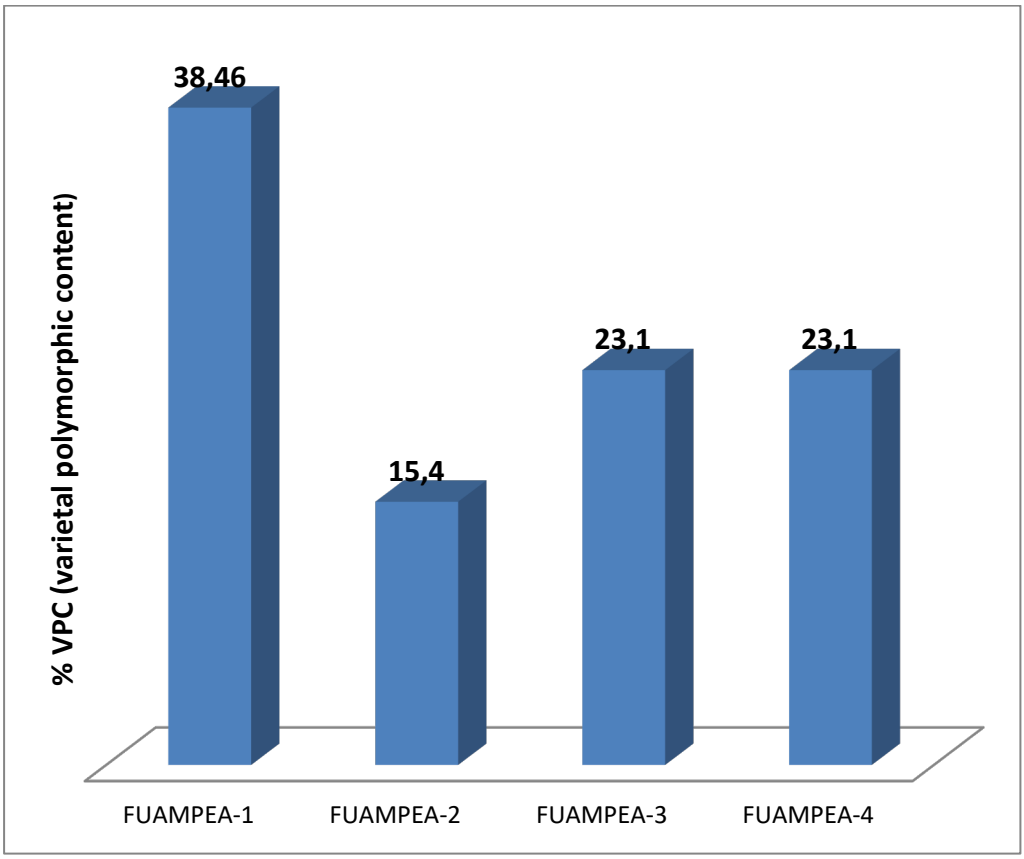


Figure 3. Varietal Polymorphic Content

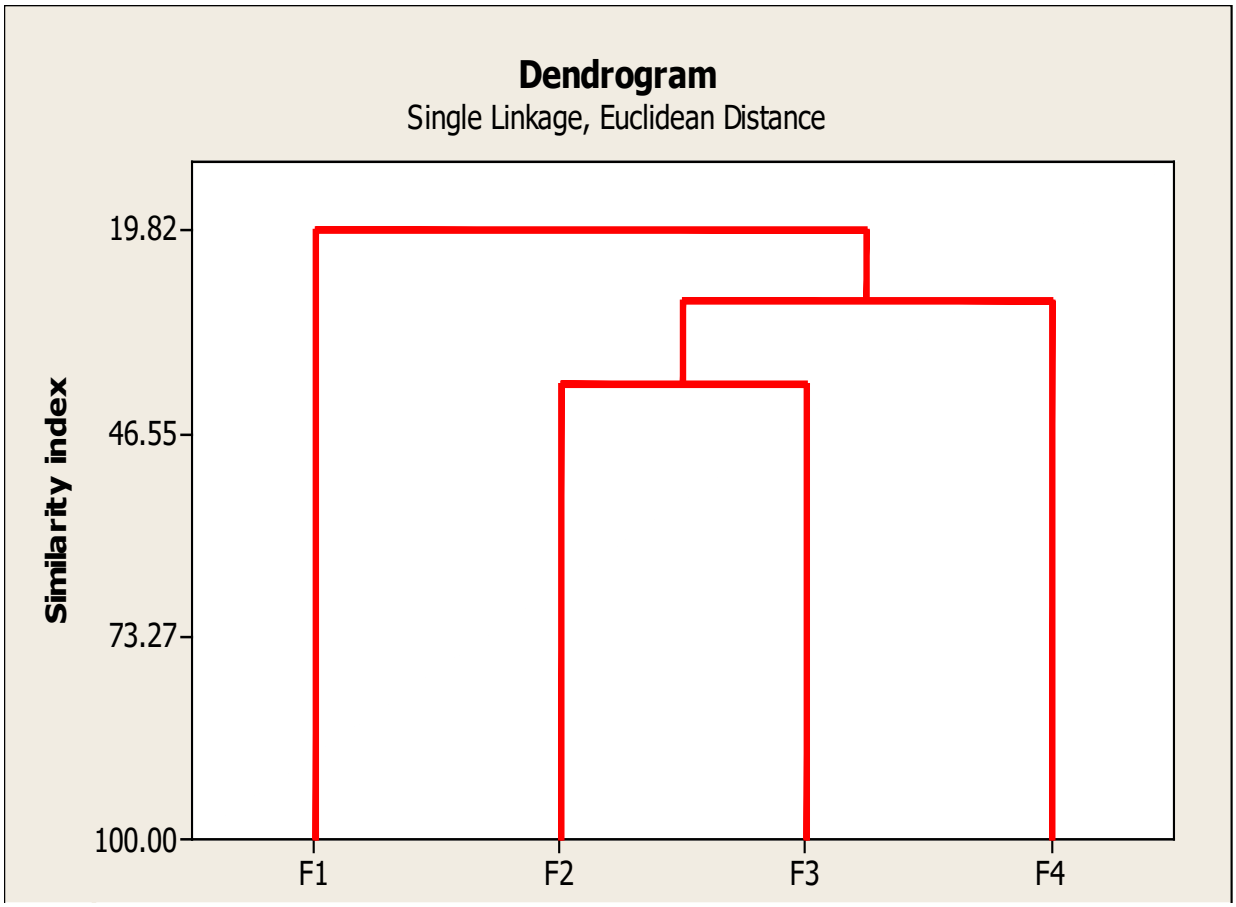


Figure 4. Phylogenetic relationship among the four cowpea varieties
Legend: FI= FUAMPEA-1; F2= FUAMPEA-2; F3= FUAMPEA-3; F4= FUAMPEA-4

Characteristically, FUAMPEA-1 (UAM09-1055-6) and FUAMPEA-2 (UAM09-1051-1) are high yielding, early maturing varieties that are resistant to *Striga* and *Alectra* (Omoigui et al. 2015, 2016, 2017) while FUAMPEA-3 and FUAMPEA-4 were developed of recent as an improvement over the previous varieties. They are brown, big seeded medium maturing types that carry high yield and resistant properties. The present investigation has showcased FUAMPEA-1as the most genetically divergent possibly in terms of other unidentified traits or variability at the gene locus of the improved traits. Other varieties are more related as they stem out of the parental source. Thus FUAMPEA-1 is a potential genetic resource material that can used to develop more cultivars when its inherent agronomic traits are fully known and the genes identified. FUAMPEA-4 is another divergent variety that may possibly carry useful genes on its genome that should be tapped.

Conclusions

The nine SSR primers employed are highly informative but four of the markers (CLM1190, CLM0342, RB38 and RB7) are selected for cowpea improvement programmes as they had higher PIC (0.98), % polymorphism (15.1%) than other primers. FUAMPEA-1 was the most divergent and genetically diverset variety followed by FUAMPEA-4. They are potential genetic resource material that can used to develop more cultivars when they are inherent agronomic traits are fully known while the genes identified and tapped. With a robust sustainable system of cowpea genetic improvement programme, national food security is assured.

Acknowledgement
The authors duly acknowledge all stakeholders including the Federal University of Agriculture Makurdi in collaboration with IITA. The contribution for the National Variety Release Committee (NVRC) is acknowledged.

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ASPECTS OF THE BIRD FAUNA FROM CIRIC VALLEY'S (IAȘI)

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Abstract

The Ciric Valley, which is located in the northeastern region of Iași, integrates a considerable variety of habitats. Wetlands are dominant, being partially covered with compact reed beds. The other habitats are the deciduous forest, and meadows located in the northern part of the studied area. These allow the settlement of a diverse bird fauna in the area. Using the method of transects, the fixed-point observation, and sound identification, we identified 84 bird species between March 2020 and February 2021. Most species belong to the order Passeriformes (53 species), the rest of the orders having a significantly lower diversity. With the exception of the 20 sedentary species, Ciric's avifauna consisted mostly of migratory species (39 summer visitors, 8 winter visitors, 13 vagrant species), and 5 partial migrant species during the research period. Among the identified bird species are 58 breeding species and 5 possible breeding species, representing 75% of the total number of the observed bird species. During the study, we identified 2 vulnerable species (*Nycticorax nycticorax* - black-crowned night-heron and *Streptopelia turtur* - european turtle-dove), 2 endangered species (*Egretta garzetta* - small egret and *Ardea alba* – great white egret) included in The Red Book of Vertebrates in Romania, and 13 species that require special conservation measures according to the Annex 1 of the Birds' Directive.
Keywords: bird fauna, Ciric, anthropogenic impact, wetlands

Introduction

The study area is located in the northeastern part of Iași and it is composed of five artificial dam lakes formed along the Ciric river (Dorobanț, Aroneanu, Ciric II, Ciric I and Ciric III or Veneția), and their surroundings. Habitats are diverse, the natural environment including wetlands, a plantation forest, meadows, respectively surrounding human settlements. Both the construction of dams and the planting of the forest aimed to prevent the risk of flooding the city during the rainy season (Calistru et al. 2000).

The vegetation is forest-steppe type and includes hydrophilic and hygrophilous vegetation, mesophilic meadows, salt-rich grasslands, xerophilous meadows, anthropophilic vegetation, and a mixed deciduous forest (Antonescu et al.,1969, Dobrescu et al. 1958). The species that make up the reeds are *Schoenoplectus lacustris*, *Phragmites comunis*, *Typha angustifolia*, *Typha latifolia*, *Plantago major* and *Symphittum officinale*. Ciric Forest was planted by man and has an area of 252 ha. Among the numerous species of planted trees, there is a dominance of *Acer negundo*, *Quercus robur*, *Robinia pseudoacacia* and *Fraxinus excelsior* (Apăvăloaei et al. 1987). *Prunus spinosa*, *Sambucus nigra*, *Rosa canina* and others make up the shrub level, and *Lepidium ruderale*, *Taraxacum officinale* and others forms the grassy layer (Nicoară 2007, Mititelu and Vițalariu 1967).

The climate is temperate-continental with an average annual temperature of 9.3 °C, with a maximum reaching 21 °C in June and a minimum in January with -4 °C. The anual rainfalls are

Received 13 December 2022; Accepted 23 December 2022; Published Online 6 January 2023; Published 3 April 2023

533.7 millimeters (mm) in average (Gugiuman and Erhan 1987). The multiannual water temperature at the lake surface varies between 19 °C and 21 °C (Calistru et al. 2000).
Previously, six ornithological research were carried out in the Ciric valley, focused on the bird fauna of Ciric forest and dam lakes, only one regarding the bird species present in the perimeter of the all five dam lakes along the Ciric Valley (Tofănescu and Gache 2009). The very first article was published in 2004, signed by Gache, which was the reference point for the next studies in the area. A year later, Butnaru shares the preliminary results obtained from field observations in the parks and peri-urban areas of Iași, a study consolidated in 2006 with a second article. Later, in 2009, Croitoru (former Butnaru) published a monograph volume regarding the bird fauna related to the urban green areas of Iași, including the one from Ciric forest and the Ciric I – III dam lakes. Ten years later, Tofănescu and Gache conducted a study discussing the risk generated by the presence of groups of birds related to the Ciric Valley upon air traffic safety in the proximity of Iași Airport, a danger present especially during migrations. And finally, in 2020 Loghin presents an update of the list of bird species present in Ciric and analyses the impact of anthropization on the distribution of birds.
The objectives of this paper were to determine the diversity of bird species and their present status inside the habitats from the Ciric Valley. We also wanted to determine if and how our obtained data differ from the ones stated in the previously mentioned studies. Anthropogenic pressure was a permanent factor in the area, therefore it is an important element in the distribution of avifauna in the Ciric Valley. For this reason, we aimed to determine the degree of anthropogenic influence on the distribution of ornithofauna.

Period of study and Methods

The present study took place over twelve months, from March 2020 to February 2021, but we mention on-going field investigations in the area following cantitative aspects of the bird fauna. The activity took place in the perimeter of the lakes along the Ciric river, respectively the artificial dam ponds Dorobanț, Aroneanu, Ciric I, Ciric II and Veneția and their surroundings, among which we mention the edge of the nearby localities and the Ciric forest, on a radius of about 20 m from the waterfront.
As study methods, we chose the method of transects, the fixed-point observation, and sound identification for some bird species. The route covers about 9 km - from the tail of Lake Dorobanț to the Ciric III dam, as shown in the figure 1. The transect followed the eastern shore of Dorobanț and Aroneanu ponds, respectively the western shore of Ciric lakes. During the journey along the linear transect, we recorded the birds' presence on both sides of the lakes while walking and from certain observational points.
We used the sound identification based on the song characteristic of each species inside the forested areas (Ciric forest), for birds hidden in reeds (such as warbler species), in the case of the call of birds of prey on the hunting territories such as *Falco tinnunculus* - common kestrel and *Buteo buteo* - eurasian buzzard or species found in the distance, on top of trees, on poles, or near human settlements, that we could not see.
We used a Nikon Aculon 8-24x binoculars to identify the bird species and a Nikon Coolpix L840 38x camera to take photos of the birds.

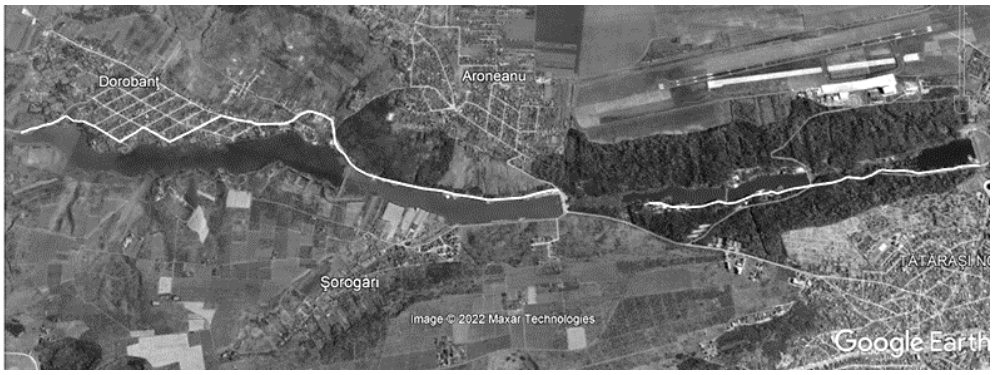

Figure 1. The transect used to cross Ciric Valley during the study

Results and discussion

Following the observations done in the field for 12 consecutive months, around the Dorobanț, Aroneanu and Ciric rezervoirs, we identified the presence of 84 bird species (table 1), taxonomically classified in 35 families belonging to 12 distinct orders. It seems that the number of bird species remains the same compared to the one mentioned by Loghin in her article but smaller compared to the 68 taxa identified by Tofănescu and Gache to which we can add the woodland typically secies that are not mentioned in their list. The reason for this might be that their on-going field investigations were made two or three times a month and they focused more on birds related to the wetlands habitats and on the migration period. During this time of the year, the birds fly in groups or flocks increasing the potential risk for the air traffic.

There are 32 species with permanent presence in Ciric are: *Ixobrychus minutus*, *Nycticorax nycticorax*, *Ardea cinerea*, *Ardea alba*, *Cygnus olor*, *Anas platyrhynchos*, *Buteo buteo*, *Accipiter gentilis*, *Gallinula chloropus*, *Chroicocephalus ridibundus*, *Larus cachinnans*, *Sterna hirundo*, *Streptopelia decaocto*, *Streptopelia turtur*, *Merops apiaster*, *Riparia riparia*, *Hirundo rustica*, *Delichon urbicum*, *Motacilla alba*, *Turdus pilaris*, *Acrocephalus scirpaceus*, *Acrocephalus arundinaceus*, *Lanius collurio*, *Pica pica*, *Corvus monedula*, *Corvus frugilegus*, *Sturnus vulgaris*, *Passer domesticus*, *Passer montanus*, *Fringilla coelebs*, *Carduelis carduelis*, and *Spinus spinus*. Unfortunately we could not met the Eurasian hoopoe (*Upupa epops*), the common swift (*Apus apus*), the common kingfisher (*Alcedo atthis*), the domestic pigeon (*Columba livia domestica*), the Eurasian coot (*Fulica atra*), the Eurasian sparrowhawk (*Accipiter nissus*) and the white stork (*Ciconia ciconia*), species mentionez in the study area during the years by other authors.

During our study, we added six new bird species to the diversity of ornithofauna of the Ciric Valley: *Galerida cristata* (the crested lark), *Luscinia luscinia* (the thrush nightingale), *Luscinia megarhynchos* (the common nightingale), *Hippolais icterina* (the icterine warbler), *Sylvia borin* (the garden warbler) and *Emberiza calandra* (the corn bunting).

The bird fauna of the Ciric valley is predominantly represented by the order Passeriformes – situation similar to the one presented by Loghin. It constitutes 63.09% of the total number of species identified in the region (53 species out of 84), bringing a considerable contribution to the taxonomic list. Charadriiformes are represented by 6 species of birds, while the orders Pelecaniformes and Piciformes by 5 species. The orders Anseriformes, Accipitriformes and Columbiformes appeared in the area each of them with 3 species, while 2 species belong to the order Podicipediformes. For the orders Falconiformes, Gruiformes, Cuculiformes and Coraciiformes we recorded a single bird species in the study area.

From the point of view of the location, we observed the higher birds' diversity in the area of Ciric I, Ciric II and Ciric III reservoirs (61 species), while we found 43 of the species d around Dorobanț reservoir and 21 in the perimeter of Aroneanu reservoir. The high level of the

diversity of bird fauna in the area of the Ciric complex could be due to the existence of two types of habitats: the wetland preferred by waterfowl species, respectively the forest where many species of passerines are established, and this explaines why the order Passeriformes is dominant for the bird fauna in the area. At the opposite pole is the Aroneanu lake with the lower recorded diversity of the bird fauna; this situation may be the consequence of the habitats' degradation by the extension of anthropogenic spaces along the eastern shore and consolidating this shore of the lake with earth, an action carried out by workers before the construction began. This activity leads to the decreasing of the surface of marsh vegetation and water surface in this sector of Ciric Valley.

Analyzing the list of the identified bird fauna, it is found that from the point of view of phenology in Romania, the summer guests are dominant in the area, represented by 41 species, being followed by the sedentary bird species (27 species), and 14 partial migrants, respectively, 8 species rarely found in winter in our country, 8 winter guests and 4 species of passage. Regarding the phenology in the study area, we mention also the dominance of summer guests with 39 species, seconded by the 20 sedentary species, 13 passage bird species, 8 winter visitors *(Anas crecca*, *Periparus ater*, *Regulus regulus*, *Turdus pilaris*, *Certhia familiaris*, *Troglodytes troglodytes*, *Spinus spinus*, *Pyrrhula pyrrhula*), and 5 partial migrant species.

The analysis of the phenological dynamics of the birds starts from the presence of the bird species in the studied area during some well-determined periods of the year. In the temperate climate area, the ecologists use a six-season division of the year, covering different phenological aspects inside the ecosystems: hibernal (November - February), prevernal (March - April), vernal (May), estival (June), serotinal (July - August) and autumnal (September - October).

The diversity of bird species varies during the six phenological aspects in the investigated area. Ciric's ornithofauna reached its peak of diversification during the serotinal season (67 species), closely followed by the estival one (66 species). During the prevernal aspect, we identified 64 species, and in the vernal one 61 bird species. In September and October of 2020 (autumnal season), we observed 51 bird species. The smallest number of species has been identified during the hibernal season (36).

Of the 84 species identified in the perimeter of the Ciric valley during March 2020 - February 2021, we recorded 58 regular breeding bird species and for another 5 we assess that they are possibly breeding species (*Nycticorax nycticorax*, *Accipiter gentilis*, *Chroicocephalus ridibundus*, *Remiz pendulinus*, *Locustella luscinioides*). In this way, we notice that 75% of the identidied bird species raise their young in the study area. During our study, we found that more bird species are nesting on the Ciric Valley comparing to the data from the previous last studies. Loghin estimated that 57 of the bird species are breeding in the area, and in 2019 Tofănescu and Gache mentioned only 41. We mention also the case of the individual *Cygnus olor* resident as non-breeding bird mentioned by Loghin, being present in the area of the Ciric I lake during our study.

Most of the observed bird species on the Ciric Valley prefer the forest habitats with less anthropogenic influence (47 species), and at the opposite pole are some that find food and shelter resources near or inside the anthropogenic spaces (17 species). The wetland habitat is also quite populated, with 27 species of birds settling here. Of all, the meadows appear as the least preferred by the birds of the Ciric bird fauna, here settling a total number of 11 species, but this habitat covers small areas in the investigated territory.

The bird fauna of the Ciric Valley is mainly negatively influenced by anthropogenic presence. The people do many activities in this region: kayaking, fishing, construction, commercial and agricultural activities, tourism and recreation. Another thing that influences the establishment of birds in the Ciric valley is its proximity to Iași International Airport. The effects of air traffic and noise produced by aircraft create discomfort for bird species and are a source of danger to

the safety of birds and people traveling on board (Tofănescu and Gache 2019). These factors explain also the presence of more bird species and specimens in the perimeter around the Veneția reservoir. Similar to the last year (Loghin 2020), we recorded more individuals of mallard (*Anas platyrhynchos*) in this sector than in the whole investigated Ciric Valley.

Consulting the Red Book of Vertebrates in Romania and the Annex 1 of the Birds Directive, we identified 14 bird species present on the Ciric Valley that appear in the lists of these documents. Two summer guests (*Nycticorax nycticorax* - black-crowned night-heron and *Streptopelia turtur* - european turtle-dove, the second one as breeding species in the north-eastern part of the Ciric forest) are vulnerable bird species in our country according to the Red Book of Vertebrates in Romania (Botnariuc and Tatole 2005), species that were also mentioned by Loghin in her study. We also identified two endangered species in the area, *Egretta garzetta* - small egret and *Ardea alba* – great white egret. Among these two, the small egret was cited before as being part of Ciric bird fauna (Tofănescu and Gache 2019), while the great white egret appears in the 2020 Loghin' study. Unfortunately, we were not able to identify other birds with conservation need such as *Ciconia ciconia, Upupa epops* and *Corvus corax*, species that appear in the 2020 list.

A number of 13 bird species of the 84 recorded species during our study on the Ciric Valley appear in the Annex 1 of Directive 2009/147/EC, which refers to species that require conservation measures of their habitat and population: *Nycticorax nycticorax*, *Ixobrychus minutus*, *Egretta garzetta*, *Ardea alba*, *Circus aeruginosus*, *Gallinago gallinago*, *Tringa glareola*, *Chlidonias hybrida*, *Sterna hirundo*, *Picus canus*, *Dendrocopos syriacus*, *Dendrocopos medius*, *Lanius collurio*, and *Lanius minor*. All these represent a percentage of 15.47% of the total identified bird fauna during our monitoring activity. The number of birds with conservation need dropped significantly (from 31 to 13) compared to the list put together by Loghin which may be a clue that the environment is changing very fast and the optimal conditions for bird fauna are decreasing in the area.

Table 1. Phenology, breeding status and distribution of Circ Valley's avifauna

No.	Species	Romania phenology	Ciric phenology	Breeding species	Observation place	Habitat
1.	*Anas platyrhynchos*	PM	PM	B	D, A, C	WL
2.	*Anas crecca*	P, WV, SV	WV	-	D	WL
3.	*Cygnus olor*	PM	P	-	C	WL
4.	*Egretta garzetta*	SV	P	-	D	WL
5.	*Ardea cinerea*	SV, RW	SV	B	D	WL
6.	*Ardea alba*	SV, RW	P	-	D	WL
7.	*Nycticorax nycticorax*	SV	SV	B?	D, A, C	WL
8.	*Ixobrychus minutus*	SV	SV	B	D, A	WL
9.	*Accipiter gentilis*	S	PM	B?	D, C	F
10.	*Circus aeruginosus*	SV, RW	SV	B	A	WL
11.	*Buteo buteo*	PM	P	-	C	F
12.	*Falco tinnunculus*	PM	P	-	C	M, F
13.	*Gallinula chloropus*	SV	SV	B	D	WL
14.	*Gallinago gallinago*	P, SV?	P	-	D	WL
15.	*Tringa glareola*	P	P	-	D	WL
16.	*Chlidonias hybrida*	SV	P	-	D	WL
17.	*Larus cachinnans*	S	PM	B	D	WL
18.	*Chroicocephalus ridibundus*	PM	SV	B?	D	WL

19.	*Sterna hirundo*	SV	SV, P	-	D, C	WL
20.	*Podiceps cristatus*	SV, RW	P	-	A	WL
21.	*Tachybaptus ruficollis*	SV, RW	P	-	A	WL
22.	*Columba palumbus*	SV, RW	SV	B	C	F, HS
23.	*Streptopelia decaocto*	S	S	B	D	HS
24.	*Streptopelia turtur*	SV	SV	B	A	F
25.	*Cuculus canorus*	SV	SV	B	D, A	F, HS
26.	*Merops apiaster*	SV	SV	B	D	M
27.	*Dendrocopos syriacus*	S	S	B	D	F, HS
28.	*Dendrocopos major*	S	S	B	C	F, HS
29.	*Dendrocopos medius*	S	S	B	C	F
30.	*Picus viridis*	S	S	B	C	F
31.	*Picus canus*	S	S	B	C	F
32.	*Oriolus oriolus*	SV	SV	B	D, A, C	F, HS
33.	*Lanius collurio*	SV	SV	B	D, C	M
34.	*Lanius minor*	SV	SV	B	D	M
35.	*Garrulus glandarius*	S	S	B	C	M
36.	*Pica pica*	S	S	B	D, A, C	M
37.	*Corvus frugilegus*	S, WV	S	B	D, C	F, HS
38.	*Corvus monedula*	S	S	B	D, C	F, M
39.	*Corvus cornix*	S	S	B	D,C	M, HS
40.	*Parus major*	S	S	B	D, A, C	F, HS
41.	*Cyanistes caeruleus*	S	S	B	D, A, C	F, HS
42.	*Poecile palustris*	S	S	B	D, A, C	F, WL
43.	*Periparus ater*	S	WV	-	D, A, C	F
44.	*Remiz pendulinus*	PM	SV	B?	D, A, C	WL
45.	*Regulus regulus*	PM, WV	WV	-	D, A, C	F
46.	*Galerida cristata*	S	SV	B	A	M
47.	*Hirundo rustica*	SV	SV	B	D, A, C	HS
48.	*Delichon urbicum*	SV	SV	B	D, C	HS
49.	*Riparia riparia*	SV	SV	B	C	M, WL
50.	*Acrocephalus arundinaceus*	SV	SV	B	D, A, C	WL
51.	*Acrocephalus scirpaceus*	SV	SV	B	D, A, C	WL
52.	*Hippolais icterina*	SV	SV	B	C	F
53.	*Locustella luscinioides*	SV	SV	B?	D	WL
54.	*Phylloscopus collybita*	SV	SV	B	C	WL
55.	*Sylvia atricapilla*	SV	SV	B	C	F
56.	*Sylvia borin*	SV	SV	B	C	F
57.	*Sylvia communis*	SV	SV	B	C	F
58.	*Sylvia curruca*	SV	SV	B	C	F
59.	*Luscinia megarhynchos*	SV	SV	B	A, C	F
60.	*Luscinia luscinia*	SV	SV	B	C	F
61.	*Muscicapa striata*	SV	SV	B	C	F, WL
62.	*Phoenicurus phoenicurus*	SV	SV	B	C	F
63.	*Phoenicurus ochruros*	SV	SV	B	C	F, HS
64.	*Erithacus rubecula*	SV, RW	SV	B	C	F

65.	*Turdus merula*	PM	S	B	C	F
66.	*Turdus philomelos*	SV	SV	B	C	F
67.	*Turdus viscivorus*	PM	P	-	C	F
68.	*Turdus iliacus*	P	P	-	C	F
69.	*Turdus pilaris*	PM, WV	WV	-	C	F
70.	*Sturnus vulgaris*	PM	SV	B	D, C	HS
71.	*Sitta europaea*	S	S	B	C	F
72.	*Certhia familiaris*	S	WV	-	C	F
73.	*Troglodytes troglodytes*	SV, RW	WV	-	C	F
74.	*Passer domesticus*	S	S	B	D, C	HS
75.	*Passer montanus*	S	S	B	D, C	HS, M
76.	*Motacilla alba*	SV	SV	B	C	WL
77.	*Carduelis carduelis*	S, WV	S	B	D, C	F, HS
78.	*Fringilla coelebs*	PM	S	B	C	F
79.	*Spinus spinus*	PM, WV	WV	-	C	F
80.	*Coccothraustes coccothraustes*	S	S	B	C	F
81.	*Pyrrhula pyrrhula*	S	WV	-	C	F
82.	*Chloris chloris*	S	SV	B	C	F
83.	*Emberiza calandra*	PM	SV	B	D	F, M
84.	*Emberiza citrinella*	S	S	B	D, C	F

Legends:
Phenology status: P – bird species in passage, WV – winter visitors, RW – rare winter visitors, SV – summer visitors, S – sedentary species, PM – partial migratory species:
Breeding status: B – breeding species, B? – probably breeding species;
Location in the study area: D – Dorobanț, A – Aroneanu, C – Ciric;
Suitable habitat: F – Forest, M – Meadow, WL – Wet Lands, HS – Human Settlements.

Conclusions

This study records the presence of 84 species of birds in the perimeter and the proximity of the reservoirs formed on the valley of Ciric River.
The bird fauna in the investigated perimeter reunites summer guests, and sedentary species, while the winter guests and partial migrant species present a lower diversity. During the migration time, this area becomes a significant resting and feeding place for birds, and we notice the appearance of some passage species in this territory.
Most of the species from the list of recorded bird fauna represent breeding bird species in the study area. This indicates that although the Ciric valley is exposed to high anthropogenic pressure, the birds meet favorable conditions for raising chicks there. At the same time, the permanent change of the natural landscape due to the intense anthropization gradually leads to the decrease of the number of individuals belonging to each species and the reduction of the bird fauna's diversity in the area.
Looking on the list of the bird species included in the previous studies done on the Ciric Valley (Gache 2004, Croitoru 2009 and Loghin 2020) and those recorded during our study, we found that only 32 bird species are present in the area every year. This represent less than half of the bird fauna identified in the all mentioned studies. The environment changes constantly and drastically to the point that one same species cannot finds proper conditions to establish in the same perimeter two consecutive years. This represents a serious problem because in the near future the valley of Ciric River might loses the most of its bird fauna's diversity.

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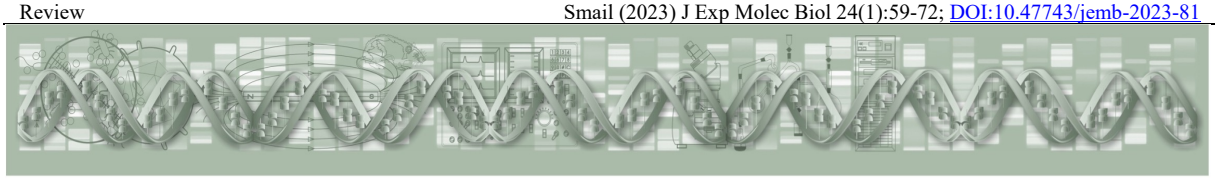

THE EPIGENETIC OF THE PANIC DISORDER

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Abstract

From this review, I discussed the epigenetics of panic disorder. Epigenetic is the changes in the heritable phenotype without any change from the DNA sequence. From one study to the next, there has been disagreement over the functions of many epigenetic processes, including DNA methylation and chromatin remodeling. The focus of the study was on how potential genes might contribute to the emergence and progression of panic disorder. Numerous candidate genes on various chromosomes, including 1q, 2p, 2q, 3, 7, 9, 11, 12q13, 12q23, and 15, may be utilized as markers in the future to diagnose panic disorder in children, are present. The fundamental value of therapies including exposure, cognitive therapy, relaxation training, and breathing retraining has not yet been determined, and recent research has not shown gene therapy's significance in treating panic disorder. Affected protection signal processing and anterior cingulate cortex-amygdala coupling can be used to distinguish between these patients to determine the effectiveness of exposure-based cognitive-behavioral therapy and associated neuroplastic alterations.
Keywords: Panic disorder, Epigenetics, candidate genes, hypermethylation, Hypomethylation, chromatin structure, and chromosomal regions

Introduction

Panic disorder is an anxiety disorder characterized by extreme, unpredictable attacks of severe fear and anticipatory anxiety, frequently comorbid with agoraphobia, with a prevalence of 1-3 percent in life (Ziegler et al. 2016). Patients provide a clinical model of stress with panic disorder. They illustrate persistent stress-related changes in sympathetic nerve biology on a good day free from a panic attack (Esler et al. 2008). Panic disorder etiopathogenesis remains largely unknown, but the risk is attributed to both genetic and environmental factors (Iurato et al. 2017). Although no clear etiology has been recognized, several factors such as genetic, environmental, neurobiological, and psychopathological variables have been proposed, like other psychiatric disorders (Kim 2018).
Epigenetics represents a change in the heritable phenotypic expression of genetic records except for changes in DNA sequence (Smail 2016, Smail 2019, Smail et al. 2022).In anxiety, affective and stress-related disorders, epigenetic mechanisms, For example, DNA methylation, have been suggested to play a vital role in the intersection of genetic and environmental factors in the pathogenesis of diseases and mediation of treatment response (Ziegler et al. 2019). Epigenetics is known to play an essential role in the etiology of complex characteristics and diseases, and one of the main types of epigenetic modifications is DNA methylation (Shimada et al. 2017, Smail et al. 2022).
For anxiety-based phenotypes, e.g., the NPSR1 gene, which has been primarily associated with panic disorder, genes related to peptide and hormone signalling have been suggested (Gottschalk et al. 2016). The Krüppel-like factor 11 (KLF11; alias TGFB-Inducible Early

Received 27 December 2022; Accepted 30 January 2023; Published Online 31 January 2023; Published 3 April 2023

Growth Response Protein 2 [TIEG2]) has been highlighted by recent studies as a novel transcriptional MAOA gene expression activator (Kollert et al. 2020). IL-4 gene methylation levels were positively linked to panic and anxiety severity, and hypermethylation of the genes CSF2, CXCL8, and IL-4 significantly correlated with higher childhood panic disorder (Zou et al. 2020).

Panic disorder symptoms

Generalized anxiety disorder (GAD), post-traumatic stress disorder (PTSD), and panic disorder (P.D.) often co-occur (Chantarujikapong et al. 2001). Intolerance to uncertainty (I.U.) was inferred as required in group samples for catastrophic interpretations. The current research explored the associations in a clinical sample between symptoms of I.U., AS, and panic disorder. Participants had a major panic disorder diagnosis, with or without agoraphobia (Carleton et al. 2014). The subsequent development of signs of fear, panic attacks, panic disorder, and anxiety characteristics (STAI-T). The ASI was the best predictor of panic symptom growth and panic attacks. The ASI was not predictive of the development of panic disorder after managing trait anxiety (Plehn et al. 2002). Popular characteristics of panic attacks include respiratory symptoms, including shortness of breath, coughing sensations, and lightheadedness. Patients with respiratory conditions, such as asthma and chronic obstructive pulmonary disease (COPD), show an elevated risk of developing the panic disorder (Mills and Searight 2020). During therapy and six months of follow-up, primary panic disorder symptoms and secondary depressive symptoms improved dramatically. The latent growth curve simulation of the parallel mechanism showed that the trajectory of depressive symptoms and the trajectory of panic disorder symptoms are significantly linked (Walderhaug et al. 2019).

No meta-analysis explicitly assesses the efficacy of Internet-based and mobile-based (IMI) treatments in adults with diagnosed panic disorder and agoraphobia (PD/A) to date (Domhardt et al. 2020). A disproportionate number of participants met the requirements for PTSD, P.D., and AUD. With 25% showing symptoms of all three conditions, co-occurrence was prevalent among detainees booked for violent offences. P.D. has emerged as the most potent single violence-related disorder, whereas the combination of PTSD, P.D., and AUD has dramatically increased the risk of violent offences (Barrett et al. 2020). In Latino populations, or comorbid asthma and panic disorder with treatment response, heart rate variability (HRV) and final tidal CO2 (ETCO2) were not studied (P.D.). The current study investigated psychophysiological variables as potential mediators of treatment response, an extension of previously published studies (Nelson et al. 2020). Results indicate that patients with a short disease span may experience substantial relief in the severity of panic symptoms during the postpartum period (Aydogan et al. 2020).

Genes related to the panic disorder

A role for miRNAs in psychiatric disorders is indicated by the involvement of microRNAs (miRNAs) in neuronal differentiation and synaptic plasticity; interaction studies and functional approaches were used to assess the effect of miRNAs on panic disorder susceptibility (Muiños et al. 2011). In people of European origin, the genetic basis of anxiety disorders overlaps with that of other psychological disorders and their intermediate phenotypes (Ohi et al. 2020). Seven SNPs that were located in or adjacent to genes, including PKP1, PLEKHG1, TMEM16B, CALCOCO1, SDK2, and CLU (or APO-J), were significantly associated with P.D. (Otowa et al. 2009).

Linkage studies have so far indicated that the transmission of panic disorder phenotypes is associated with chromosomal regions 13q, 14q, 22q, 4q31-q34, and possibly 9q31. In association studies of Panic disorder, more than 350 candidate genes have been tested, but most of these findings remain contradictory, negative, or not reproduced (Olsson et al. 2004). The

variant of SLC6A4 rs140701 polymorphism may be correlated with P.D. susceptibility, and in P.D. care, 5-HTTLPR polymorphism may be a predictor of sertraline response (Zou et al. 2020). A risk factor for Panic disorder contributing to sex-specific dysfunction in women could be polymorphisms in the CNR1 gene (Peiró et al. 2020). The TERT gene can play an essential role in the pathogenesis of accelerated ageing-related Panic disorder functional disability. These results could potentially enable us to establish new ways of predicting the outcome of panic disorder and enrich the field of telomere genetic imaging research in Panic disorder (Ding et al. 2020). A risk factor for panic disorders and phobic anxiety disorders, the CRH gene affects inhibited disposition. A significant technique for understanding the genetic basis of anxiety disorders is genetic studies of anxiety-related temperaments (Smoller et al. 2005).

Higher scores on dysthymic, cyclothymic, irritable, and nervous temperaments (Schiele et al. 2020). were correlated with less active 5-HTTLPR/rs25531 S/LG alleles. Findings help classify anxiety symptom severity biomarkers to examine startle-related genetic variants (Tomasi et al. 2020). The gene CAMKMT is located in the shared panic disorder region 2p21 (Hettema et al. 2020).

Neuropeptide S (NPS) and its cognate receptor (NPSR) molecular genetic research in humans to mediate anxiety-related actions and anxiety disorders (Domschke et al. 2011). The risk of panic disorder was increased by a variant within the respective ADORA2A gene (rs5751876) (Hohoff et al. 2010). Genetic variants of several neurotransmitter system candidate genes, each with a minor individual influence, can contribute to panic disorder susceptibility (Maron et al. 2005).

"Imaging genetics" is a groundbreaking modern research approach. Imaging genetic studies are designed to assess the effect on the cerebral function of genetic variants (polymorphisms) in regions important for P.D. In addition to supporting the importance of serotonergic and noradrenergic transmission in the etiology of P.D., recent imaging genetic studies have also shown the significance of neuropeptide S receptor, CRH receptor, channel 2 (ACCN2) gene transmembrane protein (TMEM123D), and amiloride-sensitive cation (Sobanski and Wanger 2017). As evidence from numerous studies and experimental methods points to the presumptive importance of TMEM132D in phenotypes of anxiety, some research made the decision to check whether the first association's findings might be confirmed in distinct P.D. cohorts. The top related TMEM132D SNPs from Erhardt et al. were genotyped in five additional P.D. samples obtained from the Panic Disorder International Consortium (Erhardt et al. 2012). Both a strong association between LSAMP SNPs and MDD and a possible association between LSAMP SNPs and P.D. This is the first evidence of the LSAMP gene's possible contribution to human mood and anxiety disorders (Koido et al. 2012). The ultimate goal of such investigations is to outline disease pathways and transfer this knowledge to the bedside. Such an individualized, genetically-informed approach does not only involve experiments in pharmacogenomics (for example, utilizing MAO inhibitors primarily in long allele carriers) but may also adapt the psychotherapeutic approach to the patient (Reif et al. 2014). After adaptation for age and sex, the results persisted, and there was no proof that the correlation was due to population stratification. The promoter region of the gene provided no evidence of association, 5-HTTLPR, irrespective of whether evaluated as a triallelic or biallelic locus nor did any of the other four candidate genes tested (Strug et al. 2010).

DNA methylation in the panic disorder

DNA methylation patterns have been examined for a role in anxiety disorder pathogenesis, and the role of DNA methylation-DNA methyltransferases (DNMTs) enzymes has yet to be investigated (Berking et al. 2020). Twenty-four studies documenting the relationship between antidepressants and eight genes (BDNF, MAOA, SLC6A2, SLC6A4, HTR1A, HTR1B, IL6, IL11) and whole-genome methylation resulted in a systematic literature review. The predictive

of the antidepressant response was methylation of certain sites within BDNF, SLC6A4, HTR1A, HTR1B, IL11, and the entire genome (Webb et al. 2020). Methylation of SLC6A4 may therefore constitute a potential early biomarker that predicts biologically mediated clinical changes specifically caused by the exposure (Schiele et al. 2020).

SKA2 genetic and epigenetic variation may affect the chance of developing panic disorder and the severity of its symptoms (Lisoway et al. 2020). The most promising genes for diagnosing P.D. may be COMT and SLC6A4 (Tretiakov et al. 2020). Researchers are investigating the DNA methylation epigenetic process as a result of the identification of the potential biological response mechanisms. Early research in this field has demonstrated that changes in DNA methylation may underlie the responsiveness to psychiatric therapy (Roberts et al. 2019). Because of their pivotal role in the metabolism of monoamines and as pharmacological targets of potent antidepressant drugs such as tranylcypromine, phenelzine, or moclobemide, monoamine oxidases A and B (MAOA/MAOB) are prime candidates for research into the role of DNA methylation in mental disorders (Ziegler et al. 2018). In epigenetic studies using locus-specific assays, several candidate genes (e.g., BDNF; FKBP5; SLC6A4; AVP; NR3C1; CRH; COMT; MAOA; OXTR, and APOE) for psychiatric disorders have been cited (Bortoluzzi et al. 2018)

Panic disorder patients and FOXP3 hypermethylation may potentially reflect impaired thymus and immunosuppressive Treg function in female patients with panic disorder, which may partially account for the known increased morbidity and mortality of anxiety disorders, such as cancer and cardiovascular disorders (Prelog et al. 2016). DNA hypermethylation of the NET gene promoter region. The clinical concordance is perhaps underlying and epigenetic 'comorbidity' (Esler et al. 2006). Examine the broad epigenome differences in peripheral blood in patients with P.D. Interestingly, our findings point to possible changes in panic disorder in sex-specific and functional methylation (Iurato et al. 2017). HECA hypermethylation for women with panic disorder and ASB1 hypermethylation associated with symptoms of a generalized anxiety disorder (Mufford et al. 2020). Hypermethylation of the GAD1 gene encoding the glutamic acid decarboxylase1 enzyme, the enzyme that catalyzes glutamic acid decarboxylation into GABA, has been identified. DNA hypermethylation correlates with gene expression inhibition (Peedicayil 2020). Compared with AN-Rem and NED participants, hypermethylation of a number of C.G. sites was seen in AN-Active participants (Thaler et al. 2020).

Epigenetic markers such as monoamine oxidase A (MAOA) gene DNA methylation have previously been shown to be altered in disorders linked to anxiety and stress (Schiele et al. 2020). Analysis suggests that in Caucasian patients with panic disorder, DNA hypomethylation of the 5-HTT transcriptional control region, possibly through increased serotonin transporter expression and consecutively reduced serotonin availability, could impair antidepressant treatment response (Domschke et al. 2014). Data indicate a potentially compensatory function of Hypomethylation of the GAD1 gene in panic disorder that may mediate the impact of adverse life events and rely on genetic variation (Domschke et al. 2013). The study suggests that epigenetic alterations have a potentially female-specific function, i.e., Hypomethylation of the MAO-A gene in interaction with environmental factors in panic disorder pathogenesis (Domschke et al. 2012).

Recent advances now enable the complex path of P.A.s outside the laboratory to be better tested in patients' natural environment. This will provide new insights into the fundamental processes and the effects of environmental factors that can alter gene regulation by altering the methylation of DNA (Leibold and Schruers 2018). The current pilot data do not indicate that MAO-A DNA methylation has a substantial effect on the response to antidepressant therapy. However, the current trend towards CpG-specific MAO-A gene hypomethylation, likely through increased gene expression and consecutively decreased availability of serotonin and/or

norepinephrine, may be worth following up in larger pharmacogenetic studies to potentially drive impaired antidepressant treatment response in female patients (Domschke et al. 2015). Age of DNA methylation is associated more strongly with chronological age and core psychosocial, behavioral, and health factors than relative telomere length (RTL) or copy number of mitochondrial DNA. For psychosocial and neurobehavioral factors, signals were observed for associations with epigenetic aging (Vyas et al. 2019). To further examine the predictive diagnostic value of DNA methylation reliably, longitudinal studies in animal models and patients with depression are therefore required (Chen et al. 2017). The development of genome-wide techniques capable of differentiating 5-methylcytosine (5mC) from 5hmC has shown that increasing behaviors correlate with independent disturbances of 5mC and 5hmC levels, further emphasizing the special significance of each of these brain changes (Rustad et al. 2019). Human literature is in its infancy, but it indicates some epigenetic relations with behaviors and disorders of anxiety. In particular, the effects of monoaminergic systems are seen in conjunction with findings from studies into etiology and treatment. There is also evidence that epigenetic variations may be transmitted to impact subsequent generations (Nieto et al. 2016).

Chromatin modification or chromatin remodeling in the panic disorder

At the molecular level, epigenetic mechanisms control developmental processes. Recent clinical and pre-clinical evidence obtained by ourselves and others indicates that epigenetic variations are correlated with several psychiatric disorders in different regions of the brain, including those that are stress-related (Dudley et al. 2011). Chromatin structure alteration (histone protein and DNA complexes) by acetylation (typically promoting gene expression) are epigenetic changes that are thought to affect behavioral phenotypes (Akiyoshi 2012). Transcription control closely involves the contribution of processes modifying chromatin, such as histone modification and chromatin remodeling based on ATP, but their role in pathological panic disorder is not established (Wille et al. 2016).

It has recently been found that p11 is associated with traumatic stress and depression, and the expression of the p11 gene is regulated by glucocorticoids. Two glucocorticoid response elements (GREs) in the p11 promoter region interact with the ligand-activated glucocorticoid receptor (G.R.) to up-regulate the p11 gene (Zhang et al. 2011). BAHD1 is a heterochromatinization factor that has recently been identified as a multiprotein complex component associated with HDAC1/2 histone deacetylase. BAHD1's physiological and pathophysiological functions are not well described (Pourpre et al. 2020). Histone modifications have a role in the progression of panic disorder (Wang et al. 2020). CpGenome DNA modification, the ABI Prism 7700 Sequence Detection System, and chromatin immunoprecipitation (El-Sayed et al. 2012). In high-trait anxiety mice, data show fresh evidence for localized differences in certain ATP-dependent chromatin remodeling components that may potentially result in aberrant transcriptional programs that appear as pathological anxiety (Singewald 2011). In response to stressful experiences and environmental factors, histone modification and microRNA expression could improve panic disorder (Saavedra et al. 2016).

Chromosomes of panic disorder

Some genes affect the susceptibility to pleiotropic syndrome that include panic disorder, bladder disorders, extreme headaches, mitral valve prolapse, and thyroid conditions on chromosome 13q, and likely on chromosome 22 as well (Hamilton et al. 2003). Panic disorder association studies have shown regions of interest in chromosomes 1q, 2p, 2q, 3, 7, 9, 11, 12q13, 12q23, and 15 (Logue et al. 2012). Proof that chromosome 19p13.2 contain candidate genes that contribute to the risk of panic disorder growth. Besides, the effect on other mental disorders

of the associated genes can suggest shared genetic vulnerability between mental disorders (Gregersen et al. 2016).
Amiloride-sensitive cation channel 1 (ACCN1) is located as a possible candidate gene for panic disorder on chromosome 17q11.2-q12 (Domschke et al. 2011). While several chromosomal regions, including 1q, 2q, 4q, 7p, 9q, 12q, 13q, 15q, and 22q, have been involved in linkage studies of panic disorder, they have not yet been able to establish a major gene responsible for panic disorder (Jacob et al. 2010). Several chromosome regions, including 1q, 2q, 7p, 9q, 12q, 13q, 15q, and 22q, have been involved in PD linkage studies. Candidate genes, including HTR 1A, 2A, CCK, ADORA2A, MAOA, and COMT, have been investigated in association studies (Otowa et al. 2010).

Molecular diagnosis of panic disorder

GABRB3 is associated with autism, schizophrenia, panic disorder, Asperger's syndrome, and epilepsy (OMIM 137192). CHRFAM7A (OMIM 609756) and CHRNA7 (OMIM: 118511) are linked to schizophrenia, bipolar illness, attention deficit hyperactivity disorder, Alzheimer's disease, autism, epilepsy, and learning difficulties (Chen et al. 2020). The A1 and A2a adenosine receptor (A.R.) genes differ, which influences genetic predisposition to panic disorder (Deckert et al. 1998). The polymerase chain reaction method with the study of restriction fragment-length polymorphisms (PCR-RFLP) (Inada et al. 2003). has identified HTR1A, HTR2A, and HTR2C polymorphisms. Normal diagnostic models have been used to observe linkage signals (genome-wide significance) on chromosomes 10q25, 10p12, 16q24, 16p13, and 16p12 (Cheng et al. 2006).

Treatments for panic disorder

While a great deal of research has been carried out on the effectiveness of psychiatric treatment for panic disorder with or without agoraphobia, the fundamental contribution of interventions such as exposure, cognitive therapy, relaxation training, and breathing retraining has not yet been identified (Sánchez et al. 2010). When the therapist directs the patient through the exposure activities, patients with a high behavioral propensity for active and passive avoidance react better to exposure therapy (Hamm et al. 2016). For this debilitating condition in adolescents, cognitive-behavioral therapy for panic disorder in adolescence is a feasible and potentially successful intervention (Pincus et al. 2010). Differences between these patients that assess the efficacy of exposure-based cognitive-behavioral therapy and related neuroplastic changes can be demonstrated by altered protection signal processing and anterior cingulate cortex-amygdala coupling (Lueken et al. 2013). Growing evidence indicates that cAMP response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF) are correlated with alternations under escitalopram care for patients with Panic Disorder (Yang et al. 2020). The therapeutic response for panic disorder in clinical settings is significantly less effective than it is in our imaginations. Increased data suggests that panic disorder has a chronic or remitting-relapsing clinical history (Chen and Tsai 2016).

Future of panic disorder

In the current systematic review and meta-analysis, the usefulness and efficacy of cognitive-behavioral therapy (CBT) administered via the internet on the severity of panic disorder and agoraphobia symptoms were reviewed. 27 studies have been discovered (Stech et al. 2020). The pilot study suggests that attentional bias could be investigated in future research using attention bias adjustment (ABM). A larger sample will allow the mechanisms by which ABM operates to be further studied, along with possible moderating factors and the use of psychophysiological measurements in panic disorder (Baker et al. 2020). In the COVID-19 setting, many respondents reported panic and generalized anxiety. The results indicate the need

for increased panic and generalized anxiety screening by longitudinal evaluations. During this stage (and possibly later stages) of the COVID-19 pandemic, evidence-based intervention programs and supporting resources to overcome panic and generalized anxiety seem important to Bangladeshi individuals (Islam et al. 2020). Many facts that otherwise remain isolated elements without a systematic context can be explained in perspective, i.e., the association with agoraphobia, the initiation of P.D. during puberty and young adult life, the need to be accompanied, the association with air hunger and other respiratory abnormalities, the efficacy of antidepressants and the absence of Hypothalamic-Pituitary-Adrenal (HPA) activation (HPA)(Francesetti et al. 2020). Limited focus has been given to Relapse prevention in P.D. There's a lack of recent development and definitive signs. It could be productive to re-think pharmacological research in P.D. It can help to classify accurate predictive models by collecting a wide variety of clinical and individual features/biomarkers in large-scale multicenter, long-term naturalistic studies and applying for recent technological advances (e.g., electronic medical records/'big data' platforms, wearable devices, and machine learning techniques) (Caldirola et al., 2020).

Biomarkers of panic disorder

A biomarker is characterized as an indication of normal biological processes, abnormal processes, or biological responses to a therapeutic intervention that is objectively assessed and evaluated (Cosci and Mansueto 2019).

To test the serotonergic dysfunction theory, researchers have used peripheral serotonergic indicators (such as serum serotonin levels and platelet indices). Among the platelet indices, the mean platelet volume (MPV) and platelet count have received the most research, whereas platelet distribution width (PDW) and plateletcrit have received less attention. Despite the substantial research on MPV in P.D. patients, the results are contradictory and show that MPV is a subpar diagnostic indicator. MPV is a poor biochemical marker, according to Almis et alstudy, .'s which also included a small number of individuals with generalized anxiety disorder. This finding suggests the necessity to look into other diagnostic markers (Leibold et al. 2020). Despite the fact that a lot has generally been accomplished, no perfect/specific psychiatric disease marker has yet been fixed, and medical experts concur that the search for such a marker would rank among the most difficult challenges ever encountered by researchers. The most significant factors that decrease the usefulness of potential markers include (a) the fact that some markers' levels can be affected by psychiatric disorders as well as psychiatric conditions, environmental and lifestyle factors, including stress, diet, activity level, and the use of psychoactive drugs (such as alcohol), as well as comorbidities or medications (including psychotropic disorders), and (b) incorrect or incomparable study methods (technical or material-peripheral/centra) (Waszkiewicz 2020). The results of the study suggest that the proper tapetum may act as a potential neurological marker of early sexual trauma in P.D. patients, which could make them more personally vulnerable and result in worse treatment outcomes following pharmacotherapy (Kim et al. 2020). Numerous psychiatric, psychological, physiological, sociodemographic, and lifestyle variables are associated with the progression of illness. For instance, worse outcomes at 1-year, 2-year, 6-year, and 12-year follow-ups are associated with higher baseline intensity of anxiety symptoms, the presence of somatic or psychiatric comorbidity, and higher levels of disability (Bokma et al. 2020). While the specific pathomechanism of changes in the regenerative system in mental illness is not well known, our findings suggest a disruption in regenerative processes in B.D. Patients. Stem cell activity in bipolar disorder (B.D.) varies from anxiety disorders. It also varies according to the treatment of lithium salts and other medical products. This topic needs research on peripheral blood stem cells in other mental disorders, especially depressive disorders, and also on B.D. subjects during various stages of this disorder. In the search for biomarkers helpful in the differential diagnosis

phase of mental illness, this field seems to be full of hope (Reginia et al. 2020). Results showed that people with pathological anxiety display cognitive bias in the accumulation of data, which may explain why patients with anxiety overestimate risk in their everyday lives. This explanation illustrates the value of perceptual bias measures, such as improving the clarity of desirable probabilities of outcomes (Kim et al. 2020).
In patients with MDD, greater pre-treatment levels of leptin were linked to a better response to treatment for panic symptoms, whereas higher pre-treatment levels of IL-6 were linked to a worse response to treatment for panic symptoms in patients with P.D. Even in cases where the same symptoms are seen in distinct illnesses, unique predictive biomarkers must be found, according to a variety of peripheral predictive biomarkers reported in MDD and P.D. (Kim et al. 2019). The search for biomarkers to aid in the diagnosis, prognosis, and prediction of response to therapy of psychiatric diseases is a top priority of twenty-first-century medicine. The existing method of diagnosing mental health diseases, which relies on symptom explanations rather than causal biological evidence, contributes to the current lack of biomarkers in widespread use. Along with the immense advancements in genomic, epidemiological, and neuroscience studies, new ways of conceptualizing mental health conditions educate the brain pathways and neural processes that underpin behavioral frameworks that cut through existing diagnostic structures (Pratt and Hall 2018).

Conclusions

The cause of the panic disorder is still unclear, but it may be caused by genetic, environmental, neurological, and psychopathological factors. Numerous investigations have shown that several potential genes exist for panic disorder. Epigenetic processes such as DNA methylation and chromatin remodelling play a part in how panic disorder develops. Early panic disorders can be recognized using a variety of markers on various chromosomes.

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