OPEN ACCESS

22 July, 2019
ISSN 1996-0816
DOI: 10.5897/AJPP
www.academicjournals.org

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Vol. 13(13), pp. 156-162, 22 July, 2019
DOI: 10.5897/AJPP2019.5019
Article Number: 5CDD54E61392
ISSN: 1996-0816
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African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Phytochemical analysis and investigation of sedative-hypnotic activity of methanolic stem bark extract of *Ficus abutilifolia* in mice

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Received 20 Febuary, 2019; Accepted 2 July, 2019

Most of the sedative drugs cause dose-dependent depression of the central nervous system (CNS) leading to hypnosis and possibly anaesthesia, however, these agents are associated with some side effects ranging from digestive, respiratory, immune system dysfunctions, cognitive function deterioration, tolerance, and physical dependence, hence investigations of newer and safer agents is therefore imperative. The current study sought to investigate the sedative-hypnotic effects of methanolic stem bark extract of *Ficus abutilifolia* in mice. Phytochemical constituents and sedative-hypnotic activity of the extract were investigated. Twenty Swiss albino mice were used for the experiment, the animals were randomly divided into four groups of five mice each. The mice in groups 1 and 2 were injected with the extract at the dose of 100 and 200 mg/kg, respectively, those in group 3 were injected with xylazine at the dose rate of 10 mg/kg, while the control group 4 mice received nothing at the beginning of the experiment. All the four experimental groups were injected with ketamine at the dose rate of 100 mg/kg after 30 min, all the treatments during the experimental trial were administered intraperitoneally (IP). Phytochemical analysis of the stem bark extract revealed the presence of carbohydrates, reducing sugars, cardiac glycosides, cardinolides, steroids and triterpenes, saponins, tannins, condensed tannins and flavonoids, while, anthraquinones, anthracene derivates and alkaloids were absent. The results show no significant difference ($P \geq 0.05$) on the sleep onset time between the four groups, however, statistically significant difference ($P<0.05$) was recorded in the sleep duration time between the groups The group pre-treated with low dose of the extract (100 mg/kg) before ketamine administration after 30 min exhibited longer sleeping duration time. The mice were sedated for some time after arousal from sleep. Conclusively, our finding suggests that methanolic stem bark extract of *F. abutilifolia* possess sedative-hypnotic potentials that may require further scientific elucidations.

Key words: *F. abutilifolia*, mice, stem bark extract, sedative-hypnotic activity.

INTRODUCTION

Sedatives or anxiolytic drugs reduce anxiety with little mental or motor effect. Most of the sedative drugs cause dose-dependent depression of the central nervous system (CNS) leading to hypnosis (sleep-inducing effects) and possibly anaesthesia (Kerecsen and Kirishna, 2019). These agents are however associated

with some side effects ranging from digestive, respiratory, immune system dysfunctions, cognitive function deterioration, tolerance, and physical dependence (Dhawan et al., 2003).

Dissociative anaesthetics such as ketamine are likely the most extensive and widely use class of anaesthetics in veterinary medicine (Kerecsen and Kirishna, 2019). Dissociative anaesthetics are associated with emergence delirium (e.g., anxiousness, vocalization, and thrashing). In smaller animals, this effect may be merely unpleasant but rather dangerous in larger animals like horses. This unwanted side effect can be minimized or prevented by combined administration of dissociative anesthetics with sedatives or tranquilizers agents such as xylazine, diazepam, acepromazine, etc. (Posner, 2017). Investigation of newer and safer agents with little or no side effects is therefore imperative.

Angiosperms are one of the largest plant families of which *F. abutilifolia* is a member. *F. abutilifolia* are collectively regarded as figs tree, they produce sap, leaves and flower buds (Ijeh and Ukweni, 2007). They are found mostly growing very well on rocks, bush veldts, swamps and hard surfaces (Ukwubile, 2013). Their height ranges from 21 to 50 m (Aluka, 2008) and produce flowers that are borne inside the plant between August and February yearly, a unique feature among the fig (Bouquet, 1969), propagated by seeds. The plant is distributed in Botswana, Mozambique, Nigeria, South Africa, Sudan and Zimbabwe (Berg, 1992). The plant mainly contains glycosidic phytochemical constituents such as flavonoids, saponins, anthraquinones as well as alkaloids and tannins (Ukwubile, 2013). Ethanolic leaf extract has been reported to possess antibacterial activities against *Salmonella typhi*, *Shigella dysenteriae*, and *Staphylococcus aureus* (Ukwubile, 2013). Similarly, the plant had been used traditionally in Nigeria to treat various disease conditions such as typhoid fever, dysentery and food poison (Ukwubile, 2013). The plant is said to be used by some communities as a sedative (Personal Communication, 2016).The current study therefore sought to investigate the sedative-hypnotic effects of the methanolic stem bark extract of *F. abutilifolia* in mice.

METHODOLOGY

Plant material

Freshly collected plant was identified by Mr US Gallah of National Research Institute of Chemical Technology (NARICT) Zaria, Kaduna State, Nigeria and a voucher specimen numbered 0517 was deposited at the Departmental Herbarium.

Processing and extraction

The fresh stem bark of *F. abutilifolia* was air dried under shade for two weeks, and pounded mechanically into fine particles using pestle and mortar. Five hundred grams (500 g) of the pounded dried plants materials were weighed and extracted by maceration for 72 h in 100% methanol (Pandey and Tripathi, 2014). The extracts were filtered and evaporated to dryness *in vacuo* and stored in capped bottles inside the refrigerator at 4°C until required.

Phytochemical analysis

Phytochemical screening of the stem bark extract was conducted according to the method of Evans (2009) for carbohydrate, reducing sugars, anthraquinones, anthracene derivatives, cardiac glycosides, cardinolides, saponins, tannins, condensed tannins, condensed tannins and alkaloids. Flavonoids, as described by Silva et al. (1998), triterpene and steroids as described by Harbone (1973) and Sofowora (1993). The phytochemical analysis was conducted at the Faculty of Pharmaceutical Sciences, Pharmacognosy laboratory of Ahmadu Bello University, Zaria, Nigeria.

Test for carbohydrates

Molisch's test

500 mg plant material boiled in 30 ml distilled water, filtered, 1 ml filtrate + 1 ml of Molisch's reagent +1 ml Conc. H_2SO_4. A reddish ring indicates the presence of carbohydrate" (Evans, 2009).

Fehling A and B test

1 ml of extract filtrate + 2 ml of Fehling's solution A and B + boiled for 5 min. A brick red precipitate indicates the presence of reducing sugars (Evans, 2009).

Test for tannins

Lead sub-acetate test

1 ml of extract filtrate, + 3 drops of lead-sub acetate
, a coloured precipitate indicates the presence of tannins (Evans, 2009).

Ferric chloride test

2 ml of extract filtrate + 1 ml $FeCl_3$, blue-black or greenish-black precipitate indicates tannins (Evans, 2009).

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Bromine water test

Portion of the extract filtrate + few drops of bromine water. A buff coloured precipitate indicates the presence of condensed tannins (Abubakar, 1993).

Test for saponins

Frothing test

0.5 ml of extract filtrate + 5 ml distilled water, shaken for 30 s, persistence frothing indicates saponins (Evans, 2009).

Haemolysis test

1g of the crude drug is extracted with warm water, filtered, 2 ml of 1.8% aqueous sodium chloride solution taken in two separate test tubes, to one of the test tubes 2 ml of the aqueous extract above was added, while 2 ml of distilled water was added to the second test tube. 5 drops of animal blood were added to the two test tubes and their content mixed by gentle inversion of the tubes. Haemolysis in the tube containing the crude drug and not in the control tube, indicates the presence of saponin glycosides (Brain and Turner, 1975).

Test for flavonoids

Shinoda's test

200 mg plant material in 5 ml ethanol, filtered, 1 ml filtrate + magnesium ribbon + conc. HCl a pink or red color indicates the presence of flavonoids (Silva et al., 1998).

Sodium hydroxide test

"To portion of the extract filtrate, 2ml of 10% sodium hydroxide solution was added. A yellow solution indicates the presence of flavonoids which on addition of dilute HCl becomes colourless" (Evans, 2009).

Test for steroids/terpenes

Liebermann - Burchard's

200 mg plant material in 10 ml chloroform, filtered, 2 ml filtrate + 2 ml acetic anhydride + 1 ml of conc. H_2SO_4. A blue-green ring indicates the presence of terpenes/steroids (Harborne, 1973).

Mayer's/Wagner's/Dragendorff's tests for alkaloids

200 mg plant material boiled in 20 ml of 1% H_2SO_4 in 50% ethanol, filtered, filtrate + 5 drops conc. NH_4OH + 20 ml chloroform and the two layers separated. Chloroform layer was extracted with 20 ml dilute H_2SO_4. Extract + 5 drops of Mayer's/ Wagner's/Dragendorff's reagents, a creamy/brownish-red/orangered precipitate indicates the presence of alkaloids (Evans, 2009).

Test for athraquinones and anthracene derivatives

Borntrager's test

100 mg of powdered plant in 5 ml of chloroform, filtered, 2 ml filtrate + 2 ml 10% NH_4OH. A bright pink colour indicates the presence of anthraquinones (Evans, 2009).

Modified Borntrager's test

200 mg plant material boiled in 5 ml 10% HCl, filtered). Filtrate extracted with 5 ml benzene and benzene layer shaken with 5 ml of 10% NH_4OH. A rose pink or cherry red colour indicates the presence of anthraquinone derivatives (Evans, 2009).

Test for cardiac glycosides

Keller-killiani's test

A portion of the extract dissolved in 1 ml of glacial acetic acid containing traces of ferric chloride solution; transferred into a dry test tube + 1ml of concentrated sulphuric acid added down the side of the test tube to form a lower layer at the bottom. Observed carefully at the interphase for purple-brown ring, this indicates the presence of deoxy sugars and pale green colour in the upper acetic acid layer indicates the presence of cardiac glycosides (Evans, 2009).

Kedde test

A portion of the filtrate + 1 ml of 2% solution of 3, 5 - dinitrobenzoic acid in 95% alcohol. 5% sodium hydroxide was added to make the solution alkaline, the appearance of a purple-blue colour indicates the presence of cardinolides (Evans, 2009).

Experimental animals

Twenty Swiss albino mice weighing between 16 to 26 g were used for the experiment, the animals were randomly divided into four groups (1 to 4) of five mice each. The mice were marked with picric acid solution on the head, back, tail and right leg, the fifth mouse remained unmarked for easy identification. The animals were kept in cages measuring 44 × 28 × 12.5 cm and were given access to pelleted feed and tap water *ad libitum.*

Ethical approval

Ethical permission to conduct the research was sought from the University of Abuja Ethical Committee on Animal Use (UAECAU) and was conducted in accordance with the laid down protocol.

Drugs

Ketamine hydrochloride injection USP (50 mg/ml), Rotexmedica, Trittau, Germany and Xylazine hydrochloride (20 mg/ml), XYL-M2 injection solution, VMD, Belgium were used during the experiment.

Drugs reconstitution

Ketamine 0.4 ml was diluted with 1.6 ml of distilled water to get a solution of 10 mg/ml, xylazine 0.1 ml was diluted with 1.9 ml of distilled water to get a solution of 1 mg/ml. Forty (40) and twenty 20 mg of the methanolic stem bark extract were dissolved each in 4 ml of distilled water to get solutions of 10 and 5 mg/ml, respectively.

Treatment of experimental groups

The mice in group 1 were injected with the extract at the dose rate of 100 mg/kg, group 2 mice were injected with the extract at the dose rate of 200 mg/kg, and those in group 3 were injected with xylazine at the dose rate of 10 mg/kg, while group 4 mice were not injected at the beginning of the experiment. All the experimental animal groups 1 to 4 were injected with ketamine at the dose rate of 100 mg/kg after 30 min. The drugs were administered during the investigative trials intraperitoneally (IP).

Assessment of sedative-hypnotic effects

Activities like spontaneous movement (Jiang et al., 2007) or movement in response to catch, rearing (when mouse's body inclined vertically with hind paws on the floor and forepaws on the wall of the cage (Pitychoutis et al., 2011) or when the mouse stand on its hind paws stretching the forepaws up), and climbing the wire roofing of the cage were used to assess the effects of the drugs and/or extract administered. The activities were measured with some modifications in the sense that only the presence of these activities were recorded rather than their durations. Mice in each group were observed for 3 min at 0, 30 60, 90, and 120 min for the presence of these activities. The activities were also monitored twice for every 10 min after ketamine administration, onset and duration of sleep for each mouse was recorded. Response to the drug/extract administration was graded as follows:

(1) Sleeping: Laterally recumbent and inactive (No movement at all)
(2) Awake: Sitting on four paws and inactive (No movement at all)
(3) Slightly active: Awake and moving slowly in response to catch
(4) Active: Awake, moving in response to catching and rearing
(5) Very active: Awake, moving in response to catch, rearing and climbing the wired cage roofing

Animals on grades 2 and 3 were considered sedated and slightly sedated, respectively, while, those on grades 4 and 5 were considered fully awake.

Statistical analysis

Data obtained from the study were analyzed using one-way analysis of variance (ANOVA) followed by Tukey post-hoc using of SPSS statistical software (version 4.0). Values of P ≤ 0.05 were considered statistically significant. Results are presented as mean plus/minus standard error of the mean (Mean ± SEM).

RESULTS

Phytochemical analysis of the stem bark extract of *F. abutilifolia* revealed the presence of carbohydrates, reducing sugars, cardiac glycosides, cardinolides, steroids and triterpenes, saponins, tannins, condensed tannins and flavonoids, while, anthraquinones, anthracene derivatives and alkaloids were absent (Table 1). There was no significant difference (P ≥ 0.05) in the onset of sleep time between the four different experimental groups, however, statistically significant difference (P ≤ 0.05) was recorded in the sleep duration time between the group treated with only ketamine and the other three groups that were pre-treated treated with low and high doses of the plant extract and xylazine respectively before ketamine administration after 30 min (Table 2). The group pre-treated with low dose of the plant extract (100 mg/kg), then ketamine after 30 min exhibited longer sleeping duration time, followed by the group pre-treated with high dose of the plant extract, then the xylazine pre-treated group, whereas, the group treated with only ketamine exhibited the least sleep duration time (Table 2). The plant extract, xylazine and ketamine produced a calming effect on the mice by reducing their activities and putting them in sedative condition for some period of time after arousal from sleep (Figure 1).

DISCUSSION

In present study, the phytochemical constituents recorded present in the methanolic stem bark extract of *F. abutilifolia* are in line with those reported by Ukwubile (2013). Flavonoids have been shown to have sedative effect (Aguirre-Hernández et al., 2016), so also, sedative-hypnotic activity of both flavonoids and saponins have been reported by Jiang et al. (2007). Therefore, the sedative-hypnotic effects of *F. abutilfolia* methanolic stem bark extract found in this study could be linked to the presence of phytochemical constituents like flavonoids and saponins.

The mice were very active at the beginning of the experiment before taking any form of treatment. However, their activities subsided following treatment with either the extract or the drugs, the hypnotized mice were immobilized and unable to sit on their four paws when put on lateral recumbency by the sleep was assessed with immobility and disability of the mice to be on their paws when put on lateral recumbency, no movement was seen of any form in any mice during the sleeping period except that of the abdominal muscles showing evidence of respiration. The animals did not resume their very active condition up to the end of the experiment. This finding is supported by reports from early researches on the sedative-hypnotic potentials of some plant extracts (Jiang et al., 2007; Moniruzzaman et al., 2015). Mostly, the sedative-hypnotic effects of drugs are dose-dependent, with sedation initially then followed by hypnosis and possibly anaesthesia with increasing doses of the agent in use (Kerecsen and Kirishna, 2019). In the current study, both xylazine and stem bark extract

Table 1. Phytochemical constituents of methanolic stem bark extract of *F. abutilifolia.*

Constituent	Test	Inference
Carbohydrate	Molish	+
	Fehling A and B	+
Cardiac glycoside	Keller-Killiani	+
	Kedde	+
Steroid and triterpenes	Lieberman-Buchard	+
Anthraquinone	Bontragers	-
Antracene derivatives	Modified Bontragers	-
Saponins	Frothing	+
	Haemolysis	+
Tannins	Lead sub-acetate	+
	Ferric chloride	+
	Bromine	+
Flavonoid	Shinoda	+
	Sodium hydroxide	+
Alkaloid	Mayer	-
	Dragendorff	+
	Wagners	-

Key: + Denote presence: - Denote absence.

Table 2. Effect of methanolic stem bark extract of *F. abutilifolia* on ketamine induced sleeping time in mice.

Group	Treatment	Onset of sleep (min)	Duration of sleep (min)
Group 1	100 mg/kg extract + ketamine 100 mg/kg	3.200 ± 0.200	*69.000 ± 4.60
Group 2	200 mg/kg extract + ketamine 100 mg/kg	2.800 ± 0.200	*43.000 ± 0.58
Group 3	10 mg/kg xylazine + ketamine 100 mg/kg	3.000 ± 0.320	*35.000 ± 3.90
Group 4	100 mg/g ketamine only	3 .000 ± 0.320	*20.000 ± 2.40

*: significant difference ($P < 0.05$) between control group 4 and groups 1, 2 and 3 following initial treatment with low and high doses of the extract and xylazine in groups 1, 2 and 3, respectively, then followed by ketamine administration in all the four experimental groups after 30 min.

pre-treatment prolong the sleeping time in comparison with ketamine treatment alone, however, the prolongation was not dose-dependent as the low extract dose produced the longest sleeping time. Similarly, all the drugs and plant extract induced a calming effect on the mice especially after arousal from sleep, this effect is rather termed as sedation which lasts for some period of time before the mice resumed their normal physiologic activities. Thus, the methanolic stem bark extract of *F. abutilifolia* possess sedative-hypnotic activity.

The mechanism by which the plant stem bark extract produced the sedative-hypnotic effect is not certain but most sedative-hypnotic (S-H) drugs facilitate the actions of GABA, a major inhibitory transmitter in the CNS (Kerecsen and Kirishna, 2019). $GABA_A$ receptor activation leads to increased C1 ion influx; $GABA_B$

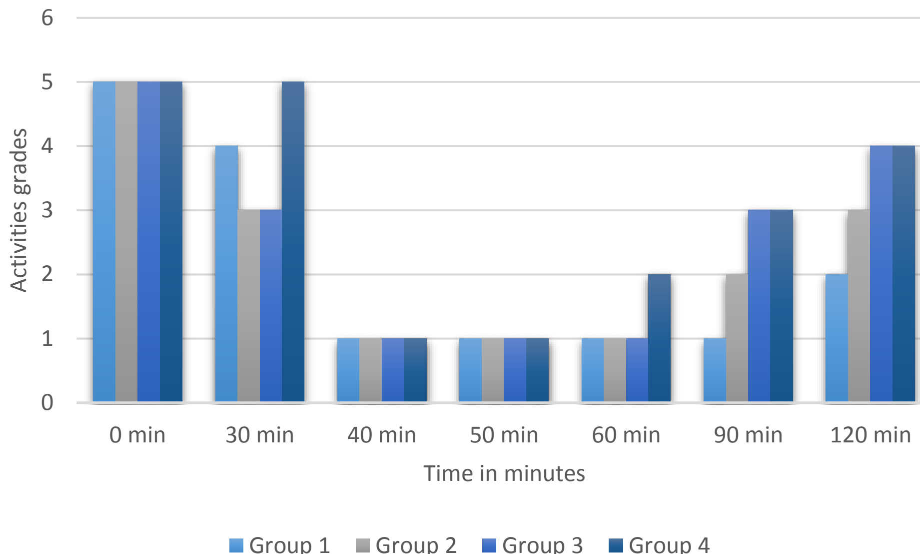

Figure 1. Sedative-hypnotic activities of methanolic stem bark extract of *F. abutilifolia* in mice. Activities grade key: 1-Sleeping; 2-Awake but sedated; 3-Awake but slightly sedated active; 4-Awake and active; 5-Awake and very active.

receptor activation causes increased efflux of K^+. Both mechanisms result in membrane hyperpolarization (Kerecsen and Kirishna, 2019), whereas, dissociative anaesthetics drugs prevent the binding of glutamate, the excitatory neurotransmitter to the NMDA receptor, resulting in depressed thalamocortical, limbic and reticular activating systems nuclei activities (Posner, 2017).

In conclusion, the current findings suggest that methanolic stem bark extract of *F. abutilifolia* possess sedative-hypnotic activity, exploration of natural products of plant origin could yield alternative sedative-hypnotic agents. Further research should be conducted to find out the active ingredient responsible for this activity and possible mechanism of action.

CONFLICT OF INTEREST

The authors have not declared any conflict of interest.

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Vol. 13(13), pp. 163-169, 22 July, 2019
DOI: 10.5897/AJPP2019.5051
Article Number: 380AE8F61468
ISSN: 1996-0816
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African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Aflatoxin M1 in cheese samples from the Amazon Region

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Received 14 May, 2019: Accepted 4 July, 2019

Aflatoxin M_1 (AFM_1) is a mycotoxin that can be found in cheeses and makes the monitoring of this food of public health interesting. In Brazil, the Amazon region has a cattle herd with dairy production of cheese, and an evaluation of the food and its risk to the population is required. The objective of this study was to evaluate the levels of AFM_1, moisture content (*mc*) and water activity (*aw*) in different cheeses types, inspected or not by the food safety authority in the Amazonas State in Brazil. All samples of coalho-type cheese were found to be in compliance with *mc* and *aw*. Both mozzarella cheeses and Minas frescal cheeses presented average *mc* below the acceptable limits. As for the samples of mozzarella cheese, these presented *aw* according to the values acceptable by the legislation, however the average of the cheese Minas frescal was inferior to the recommended values. None of the 25 cheeses samples showed AFM_1 contamination detectable by the HPLC method (LOQ = 0.0625 µg/mL). One possible explanation for the absence may be the lack of use of feed and confined animals, since in the state of Amazonas, for example, the milk-producing herd uses free grazing. Nevertheless, continuous monitoring is necessary since the consumption of cheeses produced in the state is relevant.

Key words: *Aspergillus*, water activity, moisture content.

INTRODUCTION

AFM1 is the hydroxylated metabolite of aflatoxin B1 (AFB1) and can be found in milk from animals that have ingested contaminated food by this group of aflatoxins and, consequently, in foods derived from contaminated milk (Caloni et al., 2006). It is estimated that 1 to 6% of AFB1 present in animal feed persists as AFM1 in milk and can be detected 12 to 24 h after the first AFB1 intake and reaching a high level after a few days, probably associated with the protein fraction (Battacone et al., 2003). The objective of the present study was to evaluate

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the effect of pasteurization, sterilization, preparation and storage of various dairy products (Oruc et al., 2006), and several studies have reported an increase in the concentration of this contaminant in cheeses and the elimination of water during the cheese processing (Baskaya et al., 2006; Kambar, 2006). Once formed, AFM1 is rapidly released into milk, urine and other body fluids from animals intoxicated with AFB1 (Battacone et al., 2003). The AFB1 conversion rate of food to AFM1 in milk generally ranges from 2 to 6% (Hassan et al., 2018). It can be found in milk within 12 h after the first ingestion of AFB1. After removal of the contaminated source, AFM1 disappears within 72 h. Thus, it is observed that the content of AFM1 in milk and consequently in cheese is strongly correlated with the level of AFB1 present in the raw food (Škrbić et al., 2015). AFM1 is found in milk when cattle and buffaloes receive feed contaminated with AFB1.

In several countries, monitoring and surveillance programs are being developed in order to make it possible to know the levels of contamination in milk and milk products. In Brazil, there are few studies on AFM1 in milk (Oliveira et al., 2006; Pereira et al., 2005; Garcia et al., 2003; Taveira, 2001) and derivatives such as cheeses and yoghurts (Prado et al., 2008, 2001, 2000). The occurrence of AFM1 in cheese may be due to the presence of AFM1 in milk (liquid or powder) used in the production of the product or dairy cattle may ingest food contaminated with mycotoxin or may be synthesized by fungi that grow on cheeses (López et al., 2001). In cheese manufacture, AFM1 is concentrated and retained in casein. The levels of this mycotoxin in cheeses appear to be higher in relation to milk, which is a problem of public health and worldwide concern, since cheese is a food intensively consumed by the population in general (Deveci, 2007). The fact that AFM1, a carcinogenic fungal metabolite, is present in cheeses is a fact of public health interest. In Brazil, there are about 72 types of cheeses, among which Minas Frescal cheese is the third most consumed, representing 9% of national production, followed by the so-called Mozzarella, 33% and "Prato", 24% (Marchiori, 2004). It is one of the most popular cheeses in Brazil, being consumed by all layers of the population throughout the year. It is a fresh product, for immediate consumption and of short durability. The coalho cheese is a food consumed by the local population, being part of the menu of the numerous regional cafes in the state of Amazonas. It is a source of income for some municipalities and also the food most incriminated in outbreaks that occur throughout the state, according to updated data from the Department of Epidemiological Surveillance (DVE), the Health Surveillance Foundation of Amazonas (FVS/AM). In the Amazon region in Brazil, there is production and consumption of cheeses and this requires the monitoring for future evaluation of the population risk. In the absence of studies in the North region, the objective of this study was to verify the presence and content of AFM1 in cheeses commercialized in the state of Amazonas.

MATERIALS AND METHODS

Sampling

Samples (25) of different types of cheeses (coalho, buffalo coalho, mozzarella, buffalo mozzarella and Minas frescal) were donated by the Health Surveillance Foundation (FVS). All of them came from dairy farms and cheeses from the municipalities of Autazes, Itacoatiara, Manicoré, Presidente Figueiredo and Silves, as well as samples of farms located on the AM-010 and BR-174 highways. They were sent to the capital and marketed by supermarkets of medium and large size and popular fairs of the city, from where the collections were carried out, from August to December 2017, by the fiscals of the surveillance agency. The samples were classified in two ways: (a) inspected (AI): that they had sanitary registration seal, being sold in supermarkets; and (b) not inspected (NI): samples without sanitary registration, handcrafted and sold at popular city fairs without any identification on the packaging. In total, 18 AI of coalho, buffalo coalho, mozzarella, buffalo mozzarella and Minas frescal and 07 NI all of coalho were analyzed. After being identified, they were cut into small pieces, crushed in an electric processor, stored in glass containers and refrigerated until the analyses were carried out.

Assays:

(a) Moisture content (*mc*): It was determined according to the gravimetric method by oven drying at approximately 104°C to constant weight (AOAC, 2016).
(b) Water activity (*aw*): It was determined using the AquaLab brand Water Activity Meter Dew Point 4TE by determining the dew point of the sample (AOAC, 2016).
(c) Aflatoxin M1: Separation and quantification of AFL were performed on a high performance liquid chromatography equipment (HPLC) (Shimadzu, Japan), coupled to a fluorescence detector configured with 360 nm excitation and 440 nm emission and Phenomenex® Gemini 5u C18 column 110A, 250 × 4.60 mm (5 µm). This was eluted isocratically with the mobile phase water: methanol: acetonitrile (650: 115: 270, v/v) at a flow rate of 1.0 ml/min. 50 µL of the filtered membrane filter sample (KASVI K18-430, 30 mm PES, 0.45 µm) was injected from a vial containing 500 µL. Under these conditions, the retention time was determined. All solvents used in this step were those recommended for liquid chromatography (CLAE grade) and water purified by the ultrafiltration system (MILLI - Q). From the calculation of the AFM1 peak area of the sample extract and standard solutions; it is possible to calculate the AFM1 content in the sample.
(d) Validation of the method for AFM1: Three different samples were fortified in duplicate with 1 mL of standard AFM1 solution at three concentrations: 1.0, 2.5 and 3.0 µg/mL. Subsequently, the extraction and purification were carried out with immunoaffinity column, and then the separation and quantification in HPLC. To determine the limit of detection, different concentrations of AFM1 standard solution were injected in a decreasing manner. The calibration curve was made from a 17.45 µg/mL AFM1 concentration solution in toluene and acetonitrile. From this, solutions were prepared in the following concentrations: 0.125, 0.5, 1.0, 2.5, 5.0 and 10.0 µg/mL, the range being defined. From each point of the curve, two injections were made (duplicate), obtaining the average of the readings of the same ones for the construction of

Table 1. *Aw* of the 18 samples inspected according to the type of cheese.

Cheese type	Aw[a] (mean±SD)	Limits[b]
Coalho	0.96 ± 0.00	0.91 - 0.97
Mozzarella and Buffalo Mozzarella	0.95 ± 0.01	0.93 - 0.96
Minas Frescal	0.97 ± 0.00	≥ 0.98

[a]Result obtained from the average of the samples inspected; b Limits established according to CRQ-IV (2008).

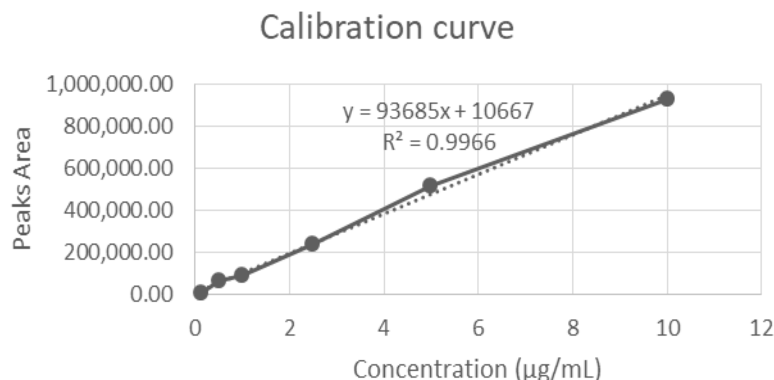

Figure 1. AFM1 calibration curve obtained by HPLC.

the calibration curve. The injection volume was 50 µL and was made from the highest to lowest concentration.

RESULTS AND DISCUSSION

Mc and Aw

Table 1 shows the results obtained in the analysis of the 18 samples of cheese inspected for aw and in Figure 1 the relation between the quantity of samples that comply with the legislation or not with respect to the *mc*.

Regarding the samples inspected, 11 of them were found in accordance with the legislation while the other 7 samples did not comply with the legislation regarding the *mc*. As for mozzarella cheese, the variation was 40.7 to 44.4%, with an average of 42.5%, being only products inspected, classifying them as medium and high *mc*. Fresh cheese showed a variation of 49 to 58.4%, with an average of 53.4%, being these products inspected, characterizing them as high and very high *mc*. Both had a mean *mc* below the acceptable limits and could be explained by the possibly inadequate transport of the samples, as high ambient temperatures could be exposed. These data are similar to those found in Matera et al. (2018) that showed Mc of 49 to 58.3%, with 60% of the samples of cheese minas frescal being outside the standard required by the legislation, that is, with a *mc* below the established levels. The *aw* of the mozzarella cheeses varied from 0.94 to 0.96 with a mean of 0.95 and the cheeses Minas frescal was 0.96 to 0.97 with a mean of 0.97, both types being inspected. The mozzarella type cheeses were in agreement with the values acceptable by the legislation, however the aw average of the cheeses Minas frescal was inferior to the accepted values, although it is not aggravating since the smaller *aw* is difficult to be growth of microorganisms, being a problem only for the sensorial characteristics of the cheese. Similar results were found by Souza et al. (2017) in which 98% of the analyzed Minas frescal cheese samples presented *aw* from 0.91, which is below the recommended value for this type of cheese.

Regarding the uncoated samples of coalho cheese, the results of water activity and *mc* were found in Table 2.

Regarding the *mc*, the curd cheeses analyzed varied from 44.9 to 51.7% to the products with inspection, and from 38.5 to 45.2% for the ones without inspection, characterizing the cheeses as being of average and high Mc. These values of *mc* are similar to the results observed in the study by Silva et al. (2010), which obtained *mc* varying from 45.5 to 51.5%, being characterized as medium to high mc cheese (39% <Mc <55%). In this work, all the samples of coalho cheese were in agreement with the values of *mc* established in the Technical Regulation of Identity and Quality of Cheeses (Brasil, 2001). Regarding the *aw* of the coalho cheeses, the variation was 0.95 to 0.96 for the cheeses with inspection and 0.94 to 0.96 for the cheeses without

Table 2. *Aw* and *mc* of the 7 samples of coalho cheese were not inspected.

Type of cheese	Aw[a] (mean±SD)	Acceptable Value[b]	*mc*[a] (mean±SD)	Acceptable Value[c]
Coalhos	0.95 ± 0.00	0.91-0.97	42.6 ± 2.47	39-55%

[a]Result obtained from the average of the 7 samples not inspected, expressed in %; [b]There are no established values for coalho cheese. From the comparison with other works one can infer the result. c Value established according to Brasil (2001) for medium to high Mc cheeses.

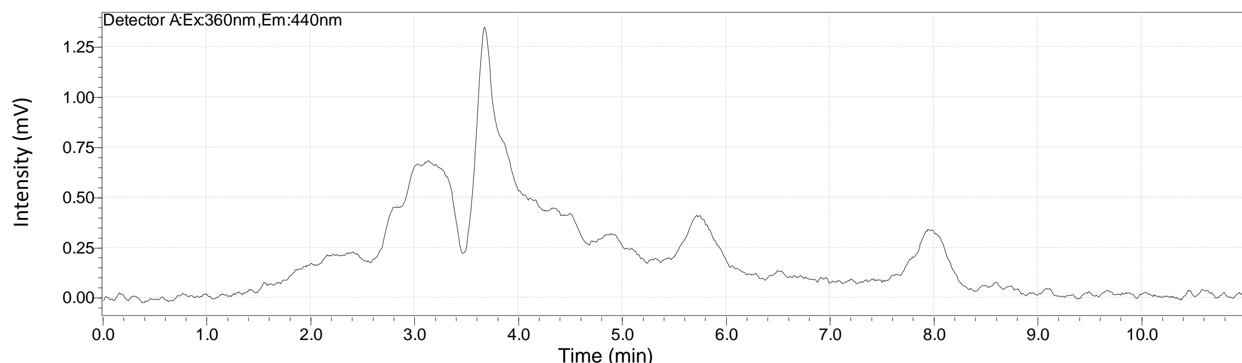

Figure 2. Chromatogram representing the limit of detection of the method for AFM1 with retention time in 7,977 min.

inspection. Andrade (2006) obtained similar aw values ranging from 0.94 to 0.97. The same author also verified that the average of *aw* for the inspected samples was superior to the one of the samples not inspected.

Determination of AFM1 in the samples

Validation of the methodology

The obtained curve correlated the area of the peaks of the chromatograms with the respective concentrations of AFM1 (Figure 1).

To determine the limit of detection, successive known concentrations of standard solution were injected decreasingly. The concentration of 0.0625 µg/mL was reached, in which the peak formation could still be observed, but it was not possible to calculate the exact area of the peak. Therefore, it was considered the detection limit of the method (Figure 2).

To determine the accuracy or recovery, the analytical methods employed in the experiment would be previously evaluated using cheeses experimentally fortified with standard AFM1 solutions at different levels. Samples were fortified with AFM1 at the required concentrations, extraction and purification were performed, and however, it was not possible to obtain recovery percentages, because during the analysis of the eluates obtained through the immunoaffinity column, there was a failure in the fluorescence module of the equipment that performs the HPLC, which has to undergo maintenance. Considering that the limit of detection was 0.0625 µg/mL and the interval determined would be sufficient to detect levels of contamination below the limit allowed by current Brazilian legislation, which establishes as maximum limit of AFM1 in cheeses, a concentration of 2.5 µg/mL, it was concluded that of the 25 samples analyzed, none of them indicated the presence of AFM1 in values higher than 0.0625 µg/mL. Therefore, the analyzed samples are in compliance with the current legislation.

The following chromatograms indicate the peak concentration 2.5 µg/mL which corresponds to the maximum concentration allowed in cheeses by legislation compared to negative sample NI 18, respectively (Figures 3 and 4).

Sylos et al. (1996), analyzed 36 samples of Minas frescal, mozzarella and cheddar cheese in Campinas, São Paulo and also the presence of AFM1 was not detected in any of the samples, although the analytical method used was thin layer chromatography (CCD) which is less sensitive than high performance liquid chromatography (HPLC). One possible explanation for the absence in all samples is that the cows and buffaloes of the Amazon region graze year-round, rarely being fed with feed. In surveys from several countries, lower levels of AFM1 in milk were observed during the summer months when larger amounts of grass were consumed or in regions where grazing periods were longer (Brown, 1982). Galvano and Galofaro (1996) also observed a seasonal trend in milk contamination by AFM1. They

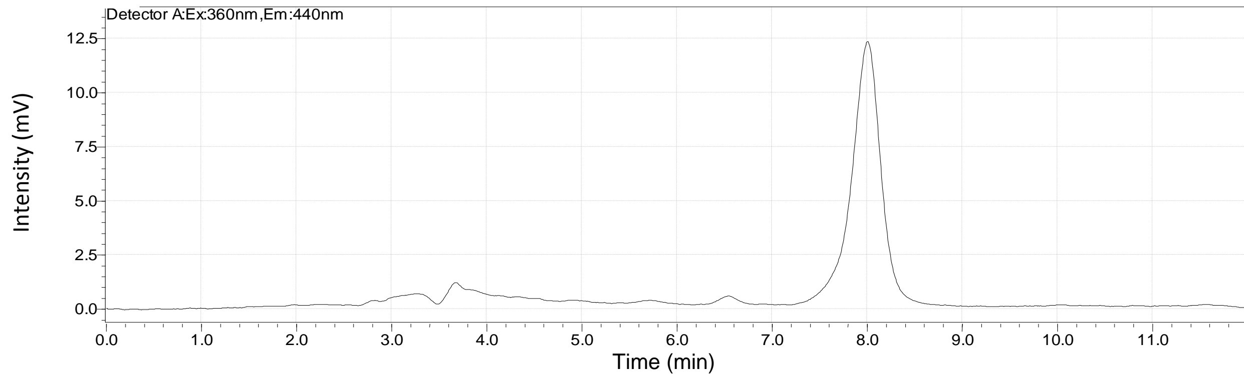

Figure 3. Chromatogram of the AFM1 standard at the concentration of 2.5 µg/mL.

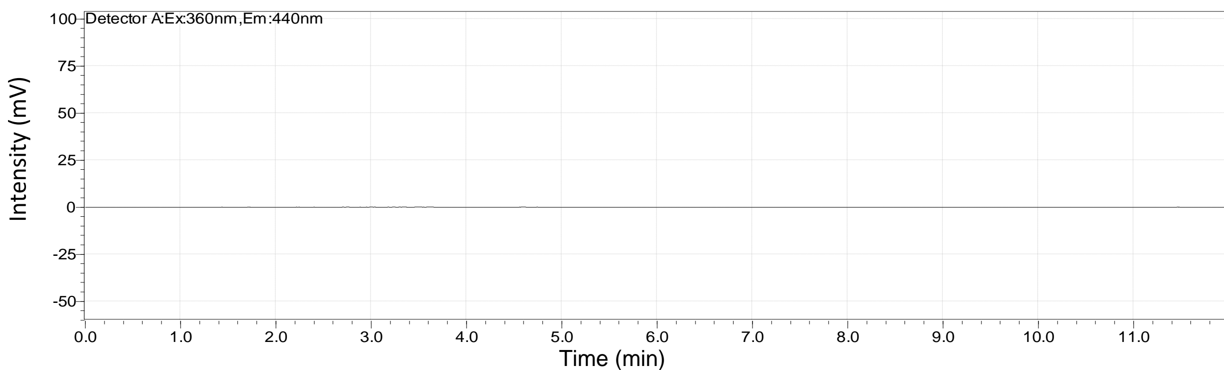

Figure 4. Chromatogram of the NI 18 sample with negative result.

found that lower rates occur during the summer months, when animals are commonly fed on pasture, unlike in winter when animals are fed by rations. Studies carried out by Embrapa (2017) show that in the north, in the rainy season, well managed pastures are capable of providing good nutrition conditions to herds conditioned to a pasture diet, requiring only mineral replacement. The reality of the Amazonian herd differs from other Brazilian states, such as the southern and southeastern regions of the country, where the animals receive rations during winter periods and the levels of contamination by AFM1 are more expressive, as evidenced in the work of Picinin et al. (2013) that evaluated AFM1 levels in raw milk samples from Minas Gerais in three different periods of the year: dry in which the rainfall is 7.9 mm and the average temperature is 19.3°C, the transition with precipitation of 100.3 mm and temperature of 20.3°C and rainfall with precipitation of 187.6 mm and temperature of 22.3°C. The authors observed higher AFM1 levels in the dry period (0.036 µg/L), followed by the transition period (0.017 µg/L) and rainy season (0.006 µg/L). The authors justified the differences due to the feeding provided to the animals in the different periods. During the dry season, supplemental feedings were provided to the livestock in feedlot. During the transition period, the cattle were kept in confinement and under pasture. However, during the rainy season, when animals are generally free for grazing, the risk of contamination has decreased (Picinin et al., 2013). It should be borne in mind that the comparison between the results of this study and those carried out by other authors is complex since the contamination of the products may vary according to locality, climatic conditions, agricultural practices adopted with the herd, among others.

Conclusion

All samples of curd cheese (AI and NI) were found to be consistent with other authors' work on *mc* and *aw*. However, there is a need for specific legislation with reference values for these variables in this type of cheese. None of the 25 samples of cheese from independent producers in the rural area of the state and

sold in supermarkets, fairs and other food establishments in the city of Manaus, showed contamination by AFM1. This is probably due to the fact that in the Amazon region, cows and buffaloes are fed exclusively on pasture all year round, not receiving feed or supplements contaminated by aflatoxins. However, it would be important to continue sampling and analysis in the following years to understand the risks related to the conditions of the different producing regions in the state of Amazonas. The absence of AFM1 contamination in the cheeses under the conditions analyzed does not rule out the continuous monitoring of this mycotoxin in the region, since the favorable conditions for the production of aflatoxins can vary according to several factors specific to our locality as the type of food given to dairy cattle. The storage conditions of the feed being offered to the milk-producing herd and the hygienic-sanitary conditions at the place of sale of the finished product must be observed in order to avoid causing other contamination.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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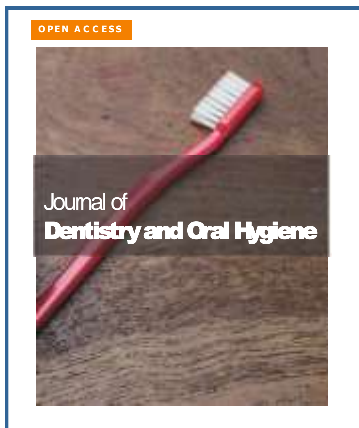

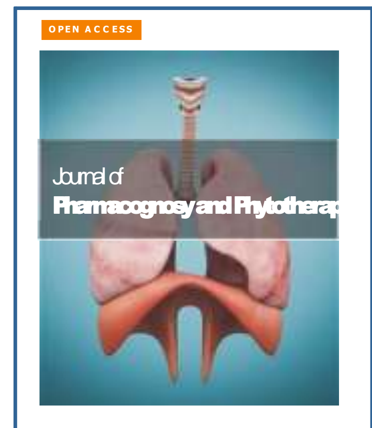

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