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In Vitro Evaluation of the Antifungal Activity of *Anogeissus leiocarpus* (DC) Guil-Per. (Combretaceae) Leaf Extracts on *Fusarium oxysporum*

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Keywords: Antifungal activities; *Fusarium oxysporum*; *Anogeissus leiocarpus*; Aqueous extract; Ethanolic extract

ABSTRACT

Introduction: Banana and oil palm crops are exposed to a fungal agent, *Fusarium oxysporum*, which is capable of causing a significant reduction in the yield of these two crops. To solve this problem, many control methods have been developed, among which, is the use of synthetic chemical products. This control method is expensive and presents risks to human health and the environment. However, the introduction of organic pesticides is a judicious and less expensive alternative for production. Objective: The objective of this study is to promote the efficacy of 70% aqueous and ethanolic extracts of *Anogeissus leiocarpus* in the control of phytopathogens. Methodology: The in vitro mycelial growth method was used in this work. Results: The antifungal tests carried out showed that the 70% ethanolic extract of *Anogeisus leiocarpus* totally inhibited the growth of *Fusarium oxyporum* fungus from the dose of 25 mg/ml. As for the aqueous extract, the highest inhibition rate was observed at 50 mg/ml. Phytochemical screening identified several chemical compounds such as sterols, tannins and polyphenols contained in these plants. Conclusion: *Anogeisus leiocarpus* is therefore a promising plant for the control of *fusarium* wilt.

INTRODUCTION

Agriculture is an essential activity for the survival of both rural and urban populations. It generates significant economic resources for producers, retailers and exporters of agricultural products. Unfortunately, this activity is confronted with numerous problems, notably poor agricultural practices and environmental constraints such as climatic hazards, soil degradation and pest pressure (Bado, 2002). The latter is marked by plant infections and diseases due mainly to fungal attacks. These phytopathogenic fungi have detrimental effects on the agricultural production of certain crops. They reduce the quantity and depreciate the quality of this production (Dabiré, 2004). They constitute a major biotic constraint for farmers as well as for the prospects of intensifying crop production (Soro *et al*., 2008; Doumbouya *et al*., 2010). They can render part or all of the crop unfit for consumption. Their actions can prevent the cultivation of a plant variety or species in a given region (Lassoudière, 2007). Synthetic fungicides are used as a means of control. However, these products have harmful effects on human health and the environment. That is, the destruction of microorganisms, the bioaccumulation of pollutants and the pollution of the water table (El Guilli, 2009). In addition, their repeated use can lead to the appearance of resistance phenomena in pests (Brent and Hollomon, 2007). Given the harmful effects of chemicals on the environment, it is necessary to move towards methods that are compatible with human health and the environment. Among these strategies, the use of medicinal plants, which contain substances to fight against these pathogens, is an approach that is increasingly sought after. In Côte d'Ivoire, according to WHO (2013), traditional medicine is experiencing unprecedented growth and is the mainstay of primary health care for the majority of the population due to its geographic, economic and cultural accessibility. However, promising new strategies involving modern biotechnology methods are being explored (Amézqueta *et al*., 2009). The exploration of natural products remains very interesting given the great diversity of antifungal compounds identified in some plant extracts (Choi *et al*., 2009). These organic compounds can be used as natural fungicides for the control of plant pathogens (Goufo *et al*., 2010). The judicious use of natural pesticides from local plants is an interesting alternative to protect crops, the environment, and living organisms (Iftikhar *et al*., 2010; Babar *et al*., 2011). These natural pesticides have already been the subject of several studies (Uddin *et al*., 2010); Katooli *et al*., 2011). Also, the antifungal properties of some plants have been proven by several authors (Bonzi, 2005; Kabore *et al*., 2007). The present study conducts an in vitro investigation on the antifungal activity of aqueous and ethanolic 70% extracts of *Anogeissus*

leiocarpus leaves on the inhibition of mycelial growth of *Fusarium oxysporum*, a major pathogen of banana and oil palm in Ivory Coast.

II-Material and Methods

II-1-Material

Plant material

The plant material consists of leaves of *Anogeissus leiocarpus* collected in the Department of Korhogo and having potentialities in the fight against fungal diseases (Fig 1).

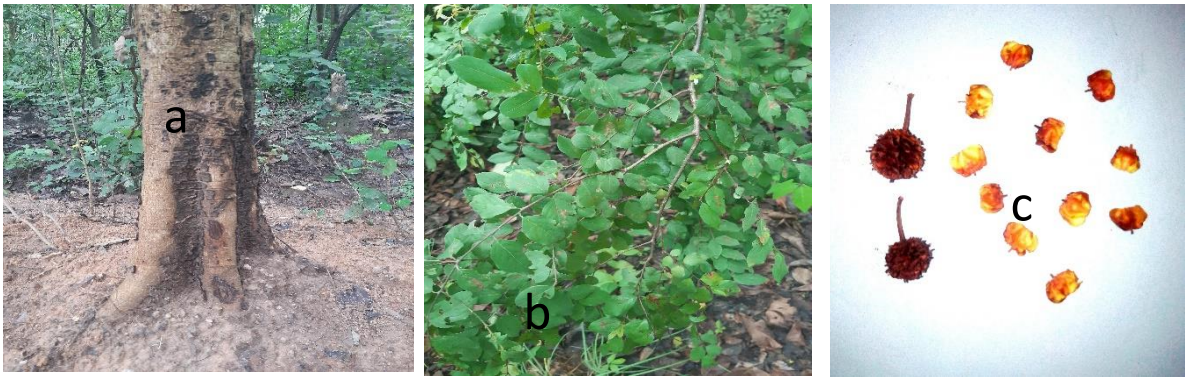

Fig 1: Different organs of *Anogeissus leiocarpus* (DC) Guil-Per (Korhogo, 2020)

a: trunk ; b: leafy branch ; c: fruits

Fungal material

The fungal material is a strain of *Fusarium oxysporum* provided by the laboratory of plant physiology of the Felix Houphouët University of Abidjan.

II-2-Methods

II-2-1-Preparation of extracts

- Total aqueous and ethanolic extracts

The fine powder of the plants obtained underwent extraction according to the method of Zirihi *et al*. (2003). For the total aqueous extract, one hundred grams (100 g) of each plant powder was added to one liter of distilled water and then homogenized in a Moulinex type "Blinder" blender for 5 to 10 minutes. The homogenate obtained was wrung out in a white

cloth square then filtered three (3) times on absorbent cotton and two (2) times on filter paper. Then, the volume of filtrate obtained was dried by evaporation in an oven at 60°C for four (4) days. The powder thus obtained constitutes the total aqueous extract. The hydroalcoholic extract was prepared according to the same procedure using a mixture of solvents comprising 70% ethanol and 30% distilled water. These extracts were weighed in order to evaluate their yield. They were then stored in sterile glass jars.

II-2-2-Calculation of the yield

The yield is determined by the ratio of the mass of the dry extract after evaporation to the mass of the dry plant material powder used for extraction (Saraka *et al*., 2019). It is expressed as a percentage and calculated according to the following formula:

$$R = m / M \times 100$$

Where R is the extraction yield; m is the mass in grams of the dry extract and M is the mass in grams of the fine powder.

II-2-3-Phytochemical screening of plant extracts

The characterization of the different chemical groups was carried out with reference to the techniques described by Békro *et al*. (2007). The phytochemical screening was carried out on the 70% aqueous and ethanolic extracts of *Anogeissus leiocarpus*

- Sterol and polyterpene screening:

Sterols and polyterpenes were searched for using the Liebermann reaction. Five (5) ml of each extract was evaporated on the sand bath. The residue was dissolved in 1 ml of acetic anhydride and 0.5 ml of concentrated sulfuric acid to triturate. The appearance, at interphase of a purple or violet ring, turning blue and then green, indicated a positive reaction indicating the presence of sterols and polyterpenes.

- Research of polyphenols:

The ferric chloride (FeCl3) reaction was used to characterize the polyphenols. To 2 ml of each extract, a drop of 2% alcoholic solution of ferric chloride was added. The appearance of a more or less dark blue-black or green coloration was a sign of the presence of polyphenols.

- Research of flavonoids:

Flavonoids were detected by the cyanidin reaction. Two (2) ml of each extract was evaporated and the residue was taken up in 5 ml of hydrochloric alcohol diluted two (2) times. On addition to 2 to 3 chips of magnesium, there is a heat release and then a pink-orange or purplish coloration. The addition of 3 drops of isoamyl alcohol intensified this coloration which confirms the presence of flavonoids.

- Research of tannins:

Tannins were detected using Stiasny's reagent. Five (5) ml of each extract was evaporated to dryness. After the addition of 15 ml of Stiasny's reagent to the residue, the mixture was kept in a water bath at 80 °C for 30 min. The observation of a coarse flake precipitate is characteristic of catechic tannins. For gallic tannins, the previous solution was filtered and then collected and saturated with sodium acetate. The addition of 3 drops of FeCl3 causes the appearance of an intense blue-black coloration, a sign of the presence of gallic tannins.

- Search for free or combined quinines:

The quinone substances were detected using Bornstraëger's reagent. A quantity of 2 ml of each extract was evaporated to dryness. The residue was triturated in 5 ml of 1/5 hydrochloric acid. The triturate was poured into a test tube and then heated in a water bath for 30 min. After cooling, it is extracted with 20 ml of chloroform. Twice diluted ammonia (0.5 ml) was added to the chloroform solution. A red or purple coloration was the sign of the presence of quinones

- Alkaloid research:

Alkaloids were characterized using Bouchardat (iodine-iodide reagent) and Dragendorff (potassium bismuthate iodine reagent) reagents. A volume of 6 ml of each solution was evaporated to dryness. The residue was taken up in 6 ml of alcohol at 60 °C. The addition of 2 drops of Dragendorff's reagent to the alcohol solution causes a precipitate or an orange coloration. The addition of 2 drops of Bouchardat's reagent to the alcoholic solution causes a reddish-brown colored precipitate and indicates a positive reaction.

- Research of saponifies :

To demonstrate the saponosides, 10 ml of the total extract was poured into a test tube. The tube was shaken for 15 and then left to stand for 15 min. A persistent foam height, greater than 1cm indicated the presence of saponosides.

III-Evaluation of the antifungal activity

III-1-Preparation of the culture medium and incorporation of the extract into the culture medium

The PDA medium amended with extracts (aqueous and/or ethanolic) was used to perform the in vitro test. Indeed, the preparation of 500 ml of PDA medium required 10 g of agar-agar, 10 g of potato mashed potato and 10 g of glucose. A range of three concentrations was selected for each aqueous (EA) and ethanolic (EE) extract. These concentrations are 50 mg/ml, 25 mg/ml and 12.5 mg/ml. They were defined according to the double dilution method of geometric bond of reason ½ (Zirihi *et al*., 2003). The different amounts of the plant extracts were incorporated into the culture media in 500 ml bottles. These media were autoclaved at 121 °C under a pressure of one bar for 30 minutes. The controls received no extracts.

III-2-Rate of inhibition of mycelial growth

Three (3) concentrations and three (3) Petri dishes per concentration were used to evaluate the fungicidal effect of the extracts. After homogenization, the different mixtures were poured into these Petri dishes, under a hood in aseptic conditions around a BUNSEN nozzle. The Petri dishes were maintained under the hood until the solidification of the medium and the seeding of the mycelial fragment. An explant was taken from the growth front of the fungus in the culture dish. The explant was placed in the center of the Petri dish on the solidified medium. The seeded Petri dishes were then sealed with adhesive film and incubated at 25 °C.

Measurement of the mean radial growth of the mycelium was performed daily in parallel with the control. This measurement was made in millimeters along two perpendicular axes drawn on the reverse side of the Petri dish. Mycelium diameters were measured until the control dishes were filled. It was done daily for seven (7) days. The rate of inhibition of mycelial radial growth was calculated according to the formula of Leroux and Credit (1978):

$$T (\%) = (D - d) / D) \times 100$$

where T is the inhibition rate; D is the mycelial growth in the control plates and d is the mycelial growth in the test plates.

III-3-Sporulation test

The evaluation of sporulation was carried out according to the following method: a washing with 5 ml of sterile distilled water of the different plates (controls and tests), which were used for the measurement of mycelial growth and which contain fungus, was carried out, to release all the spores. Then, the suspensions obtained were homogenized with a vortex. Then, these suspensions were filtered on muslin to eliminate the mycelial fragments. The number of spores for each sample was counted using a Malassez slide under the light microscope.

III-4-Statistical analysis

The data were analyzed using the methods of descriptive statistics. Data for quantitative traits were entered using an Excel spreadsheet. Statistics were compiled, followed by graphical representations. Numerical calculations and graph construction were performed with STATISTICA 7.1 and Excel 2016 software.

STATISTICA software was used to compare the average inhibition rates of the different extracts of *Fusarium oxysporum*. The average inhibition rates were calculated for each extract concentration relative to the fungal strain at the end of the seven-day incubation. The results of the inhibition test of the plant extracts on the fungal strain were expressed as percent inhibition.

An analysis of variance (ANOVA) was evaluated with the effects of the aqueous and ethanolic extracts to test the significance at the 0.05 level of a given factor from the Student's t test. When the ANOVA test was significant ($p<0.05$), a post-ANOVA Student Newman Keul (SNK) test was performed to rank the statistical units studied.

IV-Results

IV-1-Yield of extracts

Extraction of Anogeissus leiocarpus leaves provided extracts with a brown color. The yields of total extracts (aqueous and ethanolic) were 23.06% and 24.5%, respectively.

IV-2-Phytochemical screening

The phytochemical screening identified seven (7) groups of chemical compounds in the aqueous extract with the absence of gall tannins and six (6) groups of chemical compounds in the 70% ethanolic extract of *Anogeissus leiocarpus* (Table I).

Table I: Chemical compound present in the extracts of *Anogeissus leiocarpus*

Chemical group		Extract	
		Aqueous	70 % Ethanolic
Sterols		+	-
Polyphenols		+	+
Flavonoids		+	+
tannins	Catechic	+	+
	Gallic	-	+
Quinones		+	+
Alkaloids		+	+
Saponosides		+	+

+: present; -: absent

IV-3-Evaluation of the antifungal activity of *Anogeissus leiocarpus* on *Fusarium oxysporum*

IV-3-1-Rate of inhibition of mycelial growth

Observations of the effect of aqueous and ethanolic extracts on the mycelial growth of *Fusarium oxysporum* revealed a decrease in growth as a function of time and extract concentration. As the concentration of the plant extract increased in the test dishes, the growth of the fungus was slowed down compared to controls that did not contain extracts. The mycelium reached the periphery of the control dishes on day 7 of incubation (Fig 2 and 3). With the aqueous extract, the highest inhibition rate (100%) was obtained at the concentration of 50 mg/ml on *Fusarium oxysporum*. A low inhibitory power of *Anogeissus leiocarpus* was found for concentrations C2 (25 mg/ml) and C3 (12.5 mg/ ml) on the fungal strain from the 4th day of incubation. At concentration C1 (50 mg/ ml), no mycelial growth was observed. For concentrations C2 and C3, the effect of the extract progressively decreases to 97.46 and 95.65% respectively. Contrary to the aqueous extract, the ethanolic extract was more effective on the *Fusarium oxysporum* strain. 100% inhibition was observed from

concentration C2 (25 mg/ml) until the last day of incubation. For concentration C3, the effect of the extract gradually decreased to 96.95% (Fig 4 and 5).

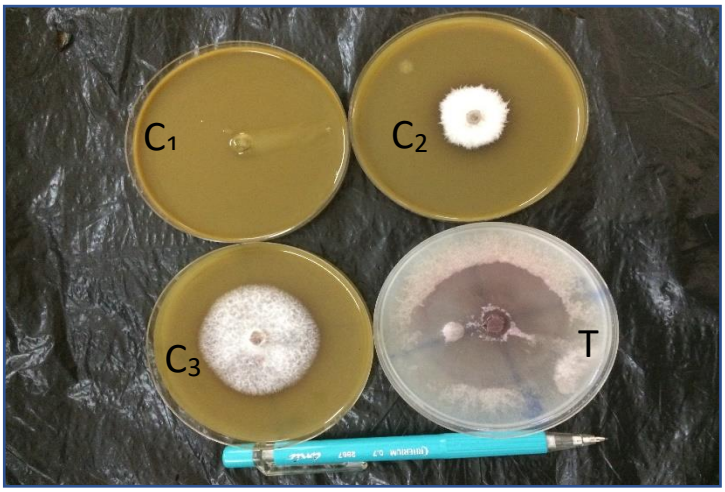

Fig 2: In vitro growth of *Fusarium oxysporum* strain in the presence of aqueous extract of *Anogeissus leiocarpus* on day 7.

C1: 50 mg/ml (no fungal growth); C2: 25 mg/ml (low fungal growth); C3: 12.5 mg/ml (medium fungal growth); T: 0 mg/ml (very high fungal growth)

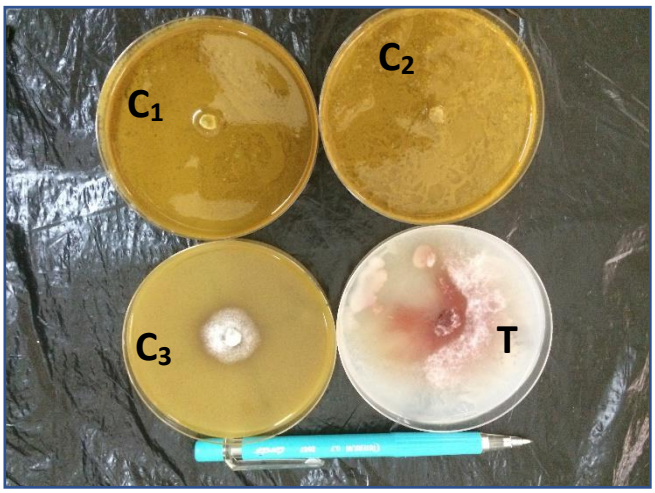

Fig 3: In vitro growth of *Fusarium oxysporum* strain in the presence of ethanolic extract of *Anogeissus leiocarpus* on day 7.

C1: 50 mg/ml (no fungal growth); C2: 25 mg/ml (no fungal growth); C3: 12.5 mg/ml (low fungal growth); T: 0 mg/ml (very high fungal growth)

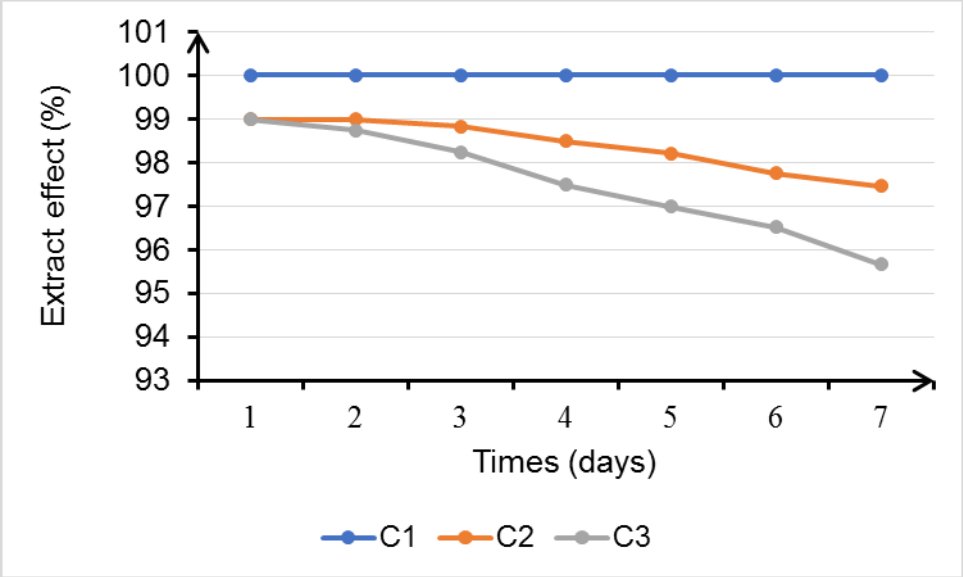

Fig 4: Effect of aqueous extract of *Anogeissus leiocarpus* on the mycelial growth of *Fusarium oxysporum* as a function of time

C1: 50 mg/ml dose; C2: 25 mg/ml dose; C3: 12.5 mg/ml dose

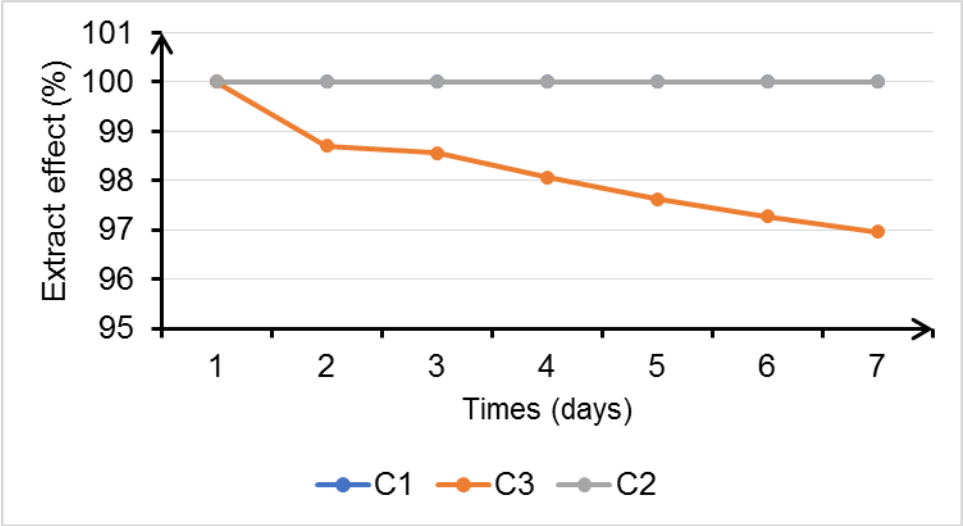

Fig 5: Effect of ethanolic extract of *Anogeissus leiocarpus* on mycelial growth of *Fusarium oxysporum* as a function of time

C1: 50 mg/ml dose; C2: 25 mg/ml dose; C3: 12.5 mg/ml dose

IV-3-2-Effect of aqueous and ethanolic extracts on sporulation of *Fusarium oxysporum*

The effect of extracts on spore production in *Fusarium oxysporum* is presented in Table II. The analysis revealed a highly significant difference between the products regarding the

number of spores produced according to the types of extracts and doses tested. For the aqueous extract, sporulation was inhibited by the 50 mg/ml concentration. However, doses C2 (25 mg/ml) and C3 (12.5 mg/ml) produced respective spore numbers of 5.06.107 and 5.15.107 which are statistically identical to each other and the control (5.75.107). As for the ethanolic type extract, spore production was highly reduced. By increasing the dose by 12.5 mg/ml, spore production was reduced by half. It decreased from 5.75.107 to 2.6.106 before being canceled at the 25 and 50 mg/ml doses.

Table II: Effect of different *Anogeissus leiocarpus* extracts on the sporulation of *Fusarium oxysporum*

Type of extracts	Doses (mg/ml)	Number of spores
Aqueous	C_1=50	0c
	C_2=25	$5,06.10^7$a
	C_3=12,5	$5,15.10^7$a
70 % Ethanolic	C_1=50	0c
	C_2=25	0c
	C_3=12,5	$2,6.10^6$ b
Control	C_0=0	$5,75.10^7$a

V-Discussion

In this study of the antifungal potency of *Anogeissus leiocarpus* on *Fusarium oxysporum*, the efficacy of the 70% aqueous and ethanolic extracts were evaluated. The 70% ethanolic extract gave a better performance compared to that of the total aqueous extract. This could be explained by the fact that the 70% ethanol certainly exalcohol-solublee alcohol soluble molecules and the water soluble small polar molecules, hence its better yield.

The antifungal activity of the extracts (aqueous and 70% ethanolic) of *Anogeissus leiocarpus* was evaluated in vitro on a strain of *Fusarium oxysporum*. A reduction in mycelial growth was observed with increasing extract concentration. This effect was more important with the 70% ethanolic extract (EE 70%) for which a 100% inhibition of mycelial growth was

obtained from the dose of 25 mg/ml, against 50 mg/ml for the aqueous extract (EA). The efficacy of the 70% ethanolic extract could be explained by a difference in chemical molecules between the aqueous extract and the 70% ethanolic extract. Indeed, secondary metabolites contained in plant extracts can influence the mycelial growth of fungi because of their qualitative and/or quantitative composition (Orsot *et al*., 2016).

Our results are similar to those of Saraka *et al*. (2019), who evaluated the antifungal activity of *Mallotus oppositifolius* (Geisel.) Müll. Arg on *Fusarium oxysporum*. Indeed, these authors showed that *Fusarium oxysporum* was sensitive to the aqueous extract from 50 mg/ml. As for the 70% ethanolic extract, they also observed a 100% inhibition rate from the 25 mg/ml dose. With the ethanolic extract, the higher inhibition rate observed could be explained by the fact that the active ingredients contained in this type of extract have a greater antifungal potential.

Ethanol is therefore better than water at extracting secondary metabolites from *Anogeissus leiocarpus*. In addition to the extracts of *Anogeissus leiocarpus* used, extracts of other plants are effective against certain phytopathogenic fungi; this is the case of aqueous extracts of *Portulaca oleracea* and *Cassia occidentalis* which effectively control the mycelial production of Bipolaris oryzae, *Curvularia* sp. and *Alternaria padwickii* (Konaté, 2006). Similarly, Kaboré et al (2007) showed the efficacy of extracts of *Azadirachta indica*, *Portulaca oleracea* and *Securidaca longepedonculata* on *Fusarium moniliforme*, *Curvularia lunata* and *Phoma sorghum*.

VI-Conclusion

The in vitro antifungal activity of *Anogeissus leiocarpus* extracts was demonstrated on *Fusarium oxysporum*. Of the two extracts, the 70% ethanolic extract exerted a strong inhibition rate on the mycelial growth of *Fusarium oxysporum*, unlike the aqueous extract. However, further work will be necessary. It will be necessary to: determine the proportions of the main groups of chemical molecules present in these extracts and if possible to carry out other in vivo tests using extracts from this plant.

Conflict of interest

The authors declare that there is no conflict of interest in this article.

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