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PHYTOCHEMICAL SCREENING AND EVALUATION OF ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF DIFFERENT PARTS OF *OLAX DISSITIFLORA* OLIVER (MUSSIRO)

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Keywords:

Olax dissitiflora, Total Phenolics, Antioxidant, Antimicrobial, Phytochemical, Mussiro

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ABSTRACT: Pharmaceutical sciences face challenges related to antibiotics toxicity and microorganism resistance. *Olax dissitiflora* (mussiro) is traditionally used in Mozambique for skin diseases treatment and beauty. The research aims to bring scientific studies for its antimicrobial and antioxidant properties. The hydroethanolic extracts of leaves, stem and stem bark of *Olax dissitiflora* species were evaluated for their phytochemical composition, antioxidant and antibacterial properties. Thus, total phenols, total flavonoids, hydrolysable and condensed tannins, total alkaloids and antioxidant activity were determined by spectrophotometric method and the correlation between the concentration and activity was made. The antibiotic properties were evaluated by disk diffusion method. The results showed higher phenolic content (159.9 ± 0.9 mg GAE / DE), and higher antioxidant activity for the stem extract in all tested methods: DPPH assay (EC_{50} = 150.80 ± 0.47 µg/mL), ABTS test (EC_{50} =25.01 ± 0.05 µg/mL), ferricyanide test (EC_{50} =23.98 ± 0.14 µg/mL) and phosphomolybdenum test (EC_{50} = 180.19 ± 0.07 µg/mL) with a very good correlation with the extract concentration. Regarding the antibacterial activity of all tested extracts, the maximum effectiveness was verified against *Salmonella typhimurium* among the three bacteria tested (*Staphylococcus aureus, Enterococcus faecalis* and *Salmonella typhimurium*). The results of this study show that the medicinal species *Olax dissitiflora* has a strong antioxidant and antibiotic action, therefore, it can be used to obtain different pharmaceutical formulations to fight inflammation, extrinsic aging and skin infections caused by bacteria or free radicals.

INTRODUCTION: Scientific and technological progress resulting from advances in pharmaceutical and medical sciences and its impact in treatment of infectious diseases is facing huge challenges resulting from development of antibiotic resistance and toxicity effects associated with the use of some medicines.

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This problem is one of the causes of countless deaths each year and is becoming more critical due to the constant increase in bacteria resistant to common antibiotics, including the drugs vancomycin, despite their role in immune modulationas mentioned by the authors [1, 2].

Studies have shown that a wide variety of medicinal plants have chemical components which have not only a role in preserving the natural defences but show also curative properties [3]. Phytoconstituents such as Phenolic compounds, alkaloids, polyphenols, carotenoids, limonoids, tocopherols, saponins, terpenoids, ascorbates and essential oils are described as the main constituents

responsible for such properties [4]. In Africa, Medicinal plants have traditionally been used for the treatment of various diseases for long time and the secondary metabolites are referred to have different mechanism and ways to act against bacteria such as the inhibition and disruption of cell wall synthesis, inhibition of DNA replication and ATP synthesis which offers a promissory alternative to be used against the multidrug resistance bacteria as descried by the authors [4, 5, 6]. Additionally, they have been used for the production of several drugs widely used in clinical practice through the isolation of their active ingredients, such as the isolation of morphine from *Papaver somniferum,* artemisin from *Artemisia annua,* codeine from opium poppy, quinine from the bark of cinchona tree which are used to fight malaria that is one of multidrug resistant disease, pilocarpine, digitoxin among other drugs identified from medicinal plants that was a great remark in medicinal and pharmaceutical field as mentioned by the author [7].*Olax dissitiflora* Oliver (Olacaceae), is a species used in Mozambique to produce a powder known as *M´siro* or N´tunkunti, used as facial mask, but also with attributed therapeutic properties. For this purpose it is used to combat the appearance of acne (pimples), facial cleansing and skin aging as defined by the author [8]. This species can be used in the treatment of hernia and sexually transmitted infections such as gonorrhoea, and wounds. It seems that it has also aphrodisiac and emetic properties and some authors report a larvacidal activity and the existence of three isolated terpenic compounds on its composition (santalbic acid, exocarpic acid and octadec-9,11-diynoic acid), which presents activity against *Anopheles arabiensis* such as mentioned by the authors [9, 10]. Studies refers to therapeutic properties of parts *Olax dissitiflora* (roots, bark and leaves) by treating respiratory diseases and these properties is attributed to the presence of secondary metabolites which are referred to have antimicrobial, anti-inflammatory and antioxidant activities such as flavonoids, saponins and alkaloids according to the authors [11, 12]. The Pharmaceutical and Cosmetic Formulation with Antimicrobial Activity, mentions the anti-inflammatory properties of saponins from *Olax dissitiflora* [13]. Although significant medicinal properties are known, there is still a lack of studies to confirm further antioxidant and antimicrobial activity of *O. dissitiflora*. This aspect motivated more studies to confirm the therapeutic properties of this species, particularly those related with facial cleansing, skin regeneration, cure of acne but also *in-vitro* antimicrobial and antioxidant properties of this medicinal species. Therefore, the present study was carried out to determine content of phenolic compound, flavonoids, tannins, total alkaloids and to evaluate the antioxidant and antimicrobial activity of *Olax dissitiflora* Oliver (mussiro) as well as the correlation with the concentration.

MATERIALS AND METHODS: The study was performed in Mozambique in 2021 at Eduardo Mondlane University. The data was obtained through experimental assays in chemistry and microbiology laboratory which were processed and then compiled for the present article. The **Table 1** presents the list of materials used in the experiment.

TABLE 1: MATERIALS USED IN THE EXPERIMENT

Test tubes	Petri dishes	Volumetric flasks	Analytical Balance
Erlenmeyer	Sterile swab	Decantation funnel	Excicator
Harmer mill	Bacteriological greenhouse	Beakers	Rotary evaporator
Oven	Bunsen burner	Vortex stirrer	UV-Vis Spectrophotometer
Orbital shaker	Filter paper	Marie bath	

Sample Preparation and Extraction: The samples used in this study were collected in Inhaca Island, Kanyaka district - Maputo province, in the south of Mozambique. Leaves and stem of *O. dissitiflora* were washed, the shell was separated from the stem, fragmented and dried in the oven at 50°C, process followed by milling using a hammer mill. The powders obtained from the leaves, stem bark and stem were used for preparation of ethanolic and hydroethanolic extracts (70:30). The extraction was performed using the maceration method under stirring (120 rpm for 72 hours) using an orbital shaker. Obtained extracts were concentrated using a Rotary evaporator and conserved for the subsequent analysis.

Preliminary Phytochemical Screening: Preliminary phytochemical screening was

performed using standard procedures described in the literature according to the authors [14, 15, 16]. With the aim of identifying main phytoconstituents (phenols, flavonoids, tannins, alkaloids, quinones, terpenes, steroids, saponins and reducing sugars) present in the extracts prepared from *Olax dissitiflora*.

Total Phenols, Flavonoids, Hydrolysable Tannins and Anthocyanin: Total phenols and total flavonoids were quantified using the Folin-Ciocalteu procedure and the aluminium chloride complexation method respectively as described by [17, 18]. Hydrolysable tannins and anthocyanins were quantified by the methods of potassium iodate and butanol-acid (ButOH-HCl) respectively, according to described by the author [19]. Determination of total phenols, total flavonoids and hydrolysable tannins were based on use of calibration curves for gallic acid, quercetin and tannic acid, respectively, and results expressed in milligrams equivalent of gallic acid, quercetin and tannic acid, per gram of dry matter, respectively, while condensed tannins were calculated using equation 1 and the results expressed in milligrams equivalent of cyanidin per gram of dry extract.

$$\text{mg CyaE/gDE} = (A \times D \times M \times V\_2)/(l \times \varepsilon \times v \times m)$$

Where by *A* represents absorbance of the sample at 530 nm; *V* the total volume of the reaction mixture (mL); *D* the dilution factor of the extract; *M* the cyanidin molar mass (g/mol); V_2 the volume of the solution of extract analysed (mL); *l* the length of sample holder (cm) and Ɛ the molar absorptivity (34.700 L/mol.cm).

Total Alkaloid Content: The determination of total alkaloids was carried out on procedures described by the author [20]. With some modifications. Five hundred mg of the hydroethanolic extract were dissolved in 10 ml of hydrochloric acid2N. The extract was filtered and transferred to a separation funnel and washed three times with 10 ml fractions of chloroform. The aqueous fraction was neutralized with sodium hydroxide 2N, followed by addition of 5 ml bromocresol green and 5 ml of phosphate buffer (pH = 4.7) and stirred until formation of a blue complex. The formed complex was extracted with different volumes of chloroform (2, 4 and 6 ml), transferred to a 10 ml volumetric flask and the volume adjusted with chloroform. Absorbance was measured at 470 nm in a UV-VIS spectro-photometer, using atropine sulphate as standard (2 – 10 µg/ml). Total alkaloids were expressed as micrograms equivalent of atropine per gram of dry extract. All tests were performed in three replicates and the results expressed as mean ± standard deviation.

Evaluation of Antioxidant Activity: Determination of antioxidant activity was performed using *in-vitro* methods based on free radical scavenging (DPPH scavenging activity and ABTS radical cation discoloration assay), reduction of ferric ion from the ferric cyanide complex $K_3[Fe(CN)_6]$ (Ferric reducing power method) and reduction of molybdenum (VI) from phosphomolybdenic acid to molybdenum (V) (Phosphomolybdenum method) described by the authors [17, 18]. The correlation was done by determining the antioxidant activity with crescent concentration of the extract.

Antimicrobial Activity: The antimicrobial activity was performed by diffusion method using microorganism concentration at 10^8 CFU/mL and different concentrations of extract on a petri plates [21]. *Olax dissitiflora* extracts were used as a source of substances with antibiotic properties against some clinically important pathogens responsible for different infections, including skin infections. The microorganisms (*S. aureus, S. typhi* and *E. faecalis*) were obtained at the Maputo Central Hospital. The cultures of the microorganisms were suspended for twenty-four hours in nutrient Broth, at standard concentrations of 0.5 McFarland, correspondent to 10^8 CFU/Ml and turbidity measured spectrophotometrically at 530 nm. Solutions of the extract and control (dimethyl sulfoxide) were sterilized using a filter with a 0.2 µm porosity (GVS Filter Technology, USA). Antibiotic activity was determined by soaking 10 µL of each extract at different concentrations (200 mg/mL – 6.25 µg/mL) onto paper discs (6 mm) and sterilized in an autoclave (121^{o}C for 15 minutes). Discs were placed in three replicates on Mueller-Hinton agar (bacteria) containing each of the microorganisms. After incubation for 24 hours, at 37^{o}C, the incubation diameter was measured using an Electronic Digital Calliper.

Statistical Analysis: Statistical analysis was performed with GraphPad Prism 9.0. The F test was used to compare data variances and the results were expressed as mean ± standard error of the mean of triplicate determinations. The statistical significance of the mean values was considered at $P < 0.05$.

RESULTS AND DISCUSSION:

Phytochemicals and Quantification: The **Table 2** presents results of the preliminary phytochemical analysis while the **Table 3** shows quantification of phenolic compounds (total phenols, total flavonoids, hydrolysable and condensed tannins) and total alkaloids respectively.

The analysis allowed detection of alkaloids, tannins, flavonoids, terpenes, steroids, quinones in all extracts studied except saponins that were not detected in ethanolic extract probably because of the absence of water as the saponins have surfactant properties by dissolving in water they form foams solutions as referred by [22].

TABLE 2: RESULTS OF THE PRELIMINARY PHYTOCHEMICAL ANALYSIS

Type of extract		Ethanolic			Hydroethanolic		
Part of the plant		Stem bark	Leaves	Stem	Stem bark	Leaves	Stem
Compounds	Phenolic compounds	+	+	+	+	+	+
	Flavonoids	+	+	+	+	+	+
	Tannins	+	+	+	+	+	+
	Alkaloids	+	+	+	+	+	+
	Terpenoids	+	+	+	+	+	+
	Steroids	+	+	+	+	+	+
	Quinones	+	+	+	+	+	+
	Reducing sugars	+	+	+	+	+	+
	Saponins	-	-	-	+	+	+

+ = Presence of the compound in the extracts; **-** = absence of the compound in the extracts.

TABLE 3: QUANTIFICATION OF PHENOLIC COMPOUNDS AND TOTAL ALKALOIDS

Hydroethanolic extract	Stem bark	Leaves	Stem
Total phenols (mg AG/gDE)	76.230 ± 0.725	83.295 ± 0.702	159.863 ± 0.906
Total flavonoids (mg Q/gDE)	18.259 ± 0.327	28.239 ± 0.320	15.461 ± 0.320
Hydrolisable tannins (mg AT/g DE)	360.960 ± 1.120	81.227 ± 0.972	844.273 ± 2.650
Condensed tannins (mg C-3-G/gDE)	1.222 ± 0.010	0.871 ± 0.010	3.421 ± 0.010
Total alkaloids (µg A/gDE)	5.489 ± 0.022	12.499 ± 0.018	6.391 ± 0.013

mg AG/gDE: milligram equivalents of gallic acid per gram of dry extract; mg Q/gDE: milligram equivalents of quercetin per gram of dry extract, mg AT/gDE: milligram equivalents of tannic acid per gram of dry extract, mg C-3-G/gDE: mg C-3-G/gDE: milligram equivalent of cyanidin-3-glucoside per gram of dry extract, µg A/gDE: microgram atropine equivalents per gram of dry extract.

Total Phenols Contents and Total Flavonoids Content: Total phenols and total flavonoids were determined in concentrations ranging from 100 - 500 µg/mL for all extracts, with correlation coefficients varying between 0.991 to 0.993 and 0.992 to 0.998 for phenols and flavonoids, respectively. The highest slope of the curve for phenols was obtained with stem extract, whereby the highest content of total phenols was obtained (159.863 ± 0.906 mg of gallic acid equivalents per gram of dry extract), while the lowest content was obtained with stem bark (76.230 ± 0.725 mg of gallic acid equivalents per gram of dry extract). Leave extract has a content of total phenols of 83.295 ± 702 mg of gallic acid equivalents per gram of dry extract. On the other side, leave extract showed the highest value of total flavonoids (28.239 ± 0.320 mg of quercetin equivalents per gram of dry extract), followed by stem bark (18.259 ± 0.327 mg of quercetin equivalents per gram of dry extract) and at last, stem 15.461 ± 0.320 mg of quercetin equivalents per gram of dry extract. All values of total flavonoids of the three extracts are significantly different at a significance level of $p<0.05$.

Hydrolysable and Condensed Tannins: Hydrolysable tannins were measured at concentrations ranging from 200 to 1000 µg/mL for all extracts, while concentration of condensed tannins ranged from 0.6 to 3 mg/mL. The lowest slope obtained for hydrolysable and condensed

tannins was obtained with stem extracts, thus suggesting the presence of higher contents in hydrolysable and condensed tannins in these extracts. Results obtained with hydrolysable tannins show significant differences ($p<0.05$) for all extracts, with values like 81.227 ± 0.972 mg of tannic acid equivalents per gram of dry extract for leave extracts, 360.96 ± 1.12 mg of tannic acid equivalents per gram of dry extract for stem bark extracts and 844.273 ± 2.650 mg of tannic acid equivalents per gram of dry extract for stem extract.

Total Alkaloids: Several studies with plant extracts such as done by the authors [23, 24, 25] mentions the key role of alkaloids in antibiotic activity of medicinal plants. Hydroethanolic extracts of the leaves exhibited the highest values (12.499 ± 0.018 µg of atropine equivalents per milligram of dry extract), when compared with stem extracts 6.391 ± 0.013 µg of atropine equivalents per milligram of dry extract) and stem bark 5.489 ± 0.022 µg of atropine equivalents per milligram of dry extract) respectively. Contents of total alkaloids obtained show significant differences for all extracts ($p<0.05$).

Antioxidant Activity: The antioxidant capacity of *Olax dissitiflora* Olivextracts were performed by using free radical scavenging methods (DPPH and ABTS methods) and reducing power methods (ferric reducing powerand phosphomolybdenum method). The **Table 4** shows the result of the antioxidant activity of all extracts using different methods. All the extracts (stem, leaves and stem bark) exhibited a strong in vitro antioxidant capacity related with the presence of secondary metabolites such as phenolic compounds (flavonoids and tannins) and other metabolites (alkaloids, terpenoids, steroids, saponins, *etc*.) previously identified and quantified in all extracts tested.

TABLE 4: ANTIOXIDANT ACTIVITY OF ALL EXTRACTS USING DIFFERENT METHODS

	EC_{50} (µg/mL)			
	Mean ± standard deviation			
Extract	**DPPH**	**ABTS**	**Ferricynide reducing power**	**Fosfomolibdenum**
Stem	150.806 ± 0.466	25.011 ± 0.053	23.976 ± 0.14	180.188 ± 0.070
Leaves	537.349 ± 1.069	58.004 ± 0.015	43.458 ± 0.013	340.688 ± 0.197
Steam bark	168.243 ± 0.278	27.442 ± 0.010	28.28 ± 0.350	340.962 ± 0.003

The stem extract was the most active in all methods performed, with the lowest EC_{50} values among all tested extracts (stem, stem bark and leaves). Values obtained for stem extract were 150.806±0.466, 25.011 ±0.053, 23.976 ± 0.14 and 180.188 ± 0.070 µg/mL for the DPPH, ABTS, ferric reducing powerand phosphomolybdenum methods, respectively. On the other hand, the EC_{50} values of the stem bark were168.243 ± 0.278, 27.442 ± 0.010, 28.28 `± 0.350 and 340.962 ± 0.003 for the DPPH, ABTS, ferric reducing power and phosphomolybdenum methods, respectively. Finally, the leaves presented EC_{50} values of 537.349 ± 1.069, 58.004 ± 0.015, 43.458 ± 0.013 and 340.688 ± 0.197 for the DPPH, ABTS, ferric reducing power (FRAP) and phosphomolybdenum methods, respectively. The best antioxidant activity exhibited by the stem extract is directly associated with the high concentration of phenolic compounds previously identified and quantified and also to the other secondary metabolites previously identified in preliminary phytochemical screening that also have contributed to the antioxidant capacity of the extracts. The correlation between the phenolic compounds and the antioxidant activity of plants extracts have been shown by many authors in their studies by using *Phragmanthera regularis* leaves, the study of phenolic compounds profile, quantitative analyses and antioxidant activity of four naked barley grains with different colours and another study about the variation in phenolic acids and antioxidant activity of navel orange at different growth ages [17, 26, 27], which gave a strong support about the relation between the antioxidant activity and the amount of phenolic compound in plants extracts. The results obtained in this study show great evidence of therapeutic properties of the aerial parts of *Olax dissitiflora* (mussiro plant) with greater emphasis on the stem that is already very traditionally used in skin treatments, therefore, these results are scientific evidence about its medicinal value and can be used to treat oxidative stress related diseases.

Skin problems including cancer,loss of elasticity, appearance of wrinkles and depigmentationare generally associated to oxidative stress generated by production of reactive oxygen species [28, 29]. This is the reason of the growing interest in the use of cosmetics products with a protective action and a capacity to delay the aging process of the skin [30]. This includes the use of natural products from a vegetable origin, in the form of extracts or isolated secondary metabolites with antioxidant activity [31, 32]. Results obtained in this study show a significant antioxidant activity with good extract concentration correlation of the stem, leaves and stem bark extracts by different methods (DPPH, $ABTS^{+}$, Ferricyanide reducing power and Phosphomolibdenium) as showed on **Fig. 1-4.**

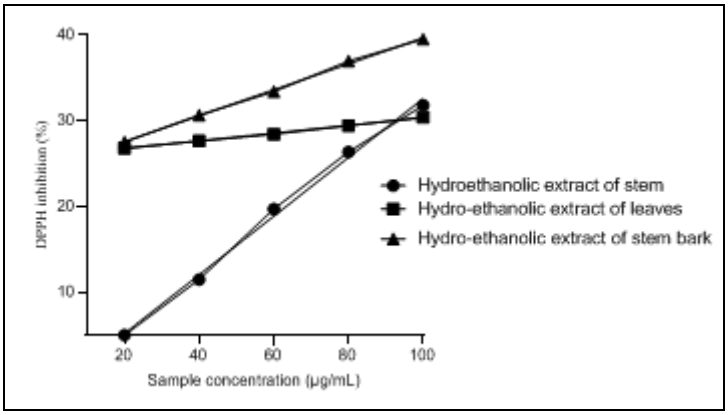

FIG. 1: CORRELATION BETWEEN CONCENTRATIONS OF THE EXTRACT WITH THE ANTIOXIDANT ACTIVITY (DPPH*)

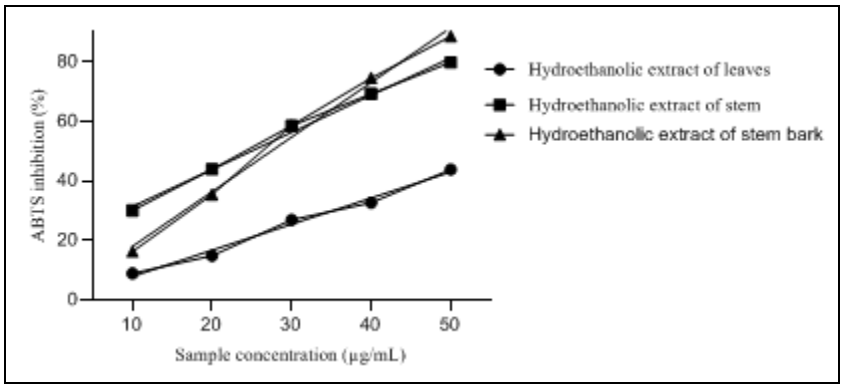

FIG. 2: CORRELATION BETWEEN CONCENTRATIONS OF THE EXTRACT WITH THE ANTIOXIDANT ACTIVITY ($ABTS^{+}$)

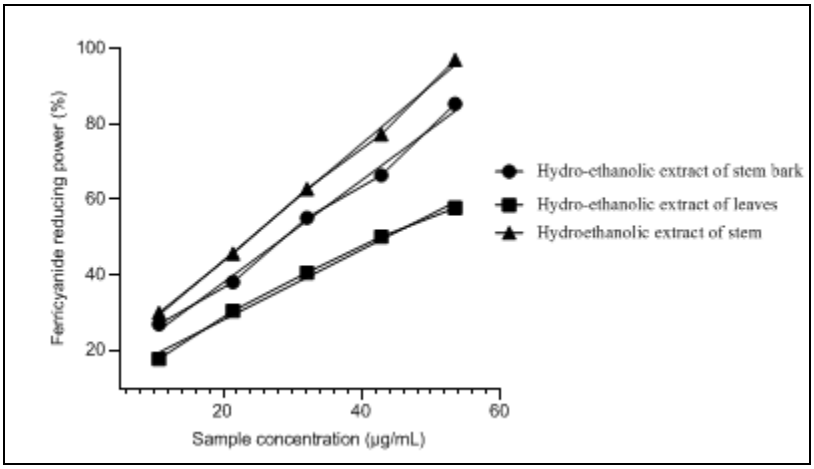

FIG. 3: CORRELATION BETWEEN CONCENTRATIONS OF THE EXTRACT WITH THE ANTIOXIDANT ACTIVITY (FERRICYANIDE REDUCING POWER)

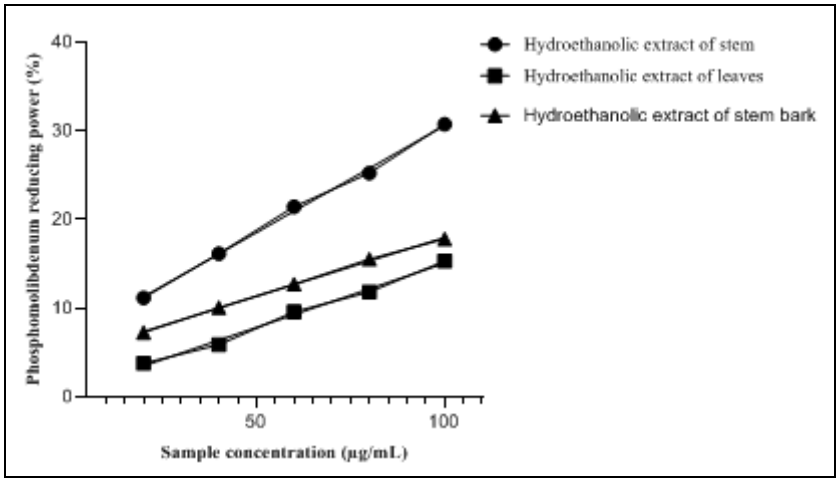

FIG. 4: CORRELATION BETWEEN CONCENTRATIONS OF THE EXTRACT WITH THE ANTIOXIDANT ACTIVITY (PHOSPHOMOLYBDENUM)

These extracts exhibited a strong *in-vitro* antioxidant activity, action associated with the secondary metabolites as phenolic compounds (flavonoids and tannins) previously identified and quantified in the three extracts. Antioxidant and antimicrobial activity observed in the different *Olax dissitiflora* extracts are explained by: (1) the presence of phenolic compounds responsible for the elimination of free radicals and inhibition of bacterial proliferation, fighting in this way against oxidative and infectious processes; (2) other metabolites as alkaloids and terpenoids that are responsible too for the antimicrobial activity of the studied extracts.

Antimicrobial Activity: Antibiotic activity has been determined for the hydroethanolic extracts of the stem, stem bark and leaves of *Olax dissitiflora*. The extracts were evaluated by Agar diffusion method against three microorganisms selected on the basis of their ability to infect skin and other soft tissues[33, 34, 35] namely, *Staphylococcus aureus*, *Salmonella typhi* and *Enterococcus faecalis*. All extracts showed activity against tested microorganisms, in almost all doses.

In the experiments with stem extract the inhibition varied in following order: *Staphylococcus aureus* (6.55 ± 0.00 - 28.36 ± 1.09 mm) < *Enterococcus faecalis* (6.55 ± 0.00 - 32.73 ± 1.09 mm) < *Salmonella typhi* (7.636 ± 0.00 - 37.09 ± 1.09 mm). However, minimum inhibitory dose was the same for all microorganisms studied (0.0625 µg/disc). The **Fig. 5** shows the correlation between the inhibition zone and the concentration of the stem extract.

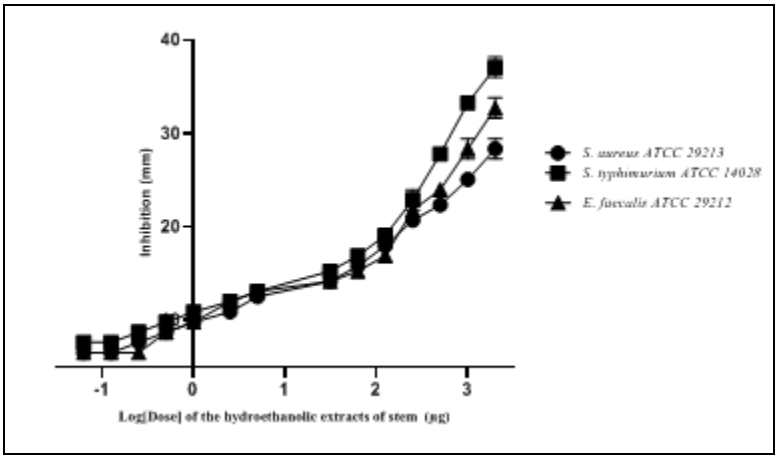

FIG. 5: INHIBITION ZONES OF THE BACTERIA IN FUNCTION OF THE STEM EXTRACT DOSE

In the experiments with stem bark extracts logarithmic curves of Staphylococcus *aureus* and *Enterococcus faecalis* show almost an overlap at maximum dose (2000 µg/disc) as showed on the **Fig. 6.**

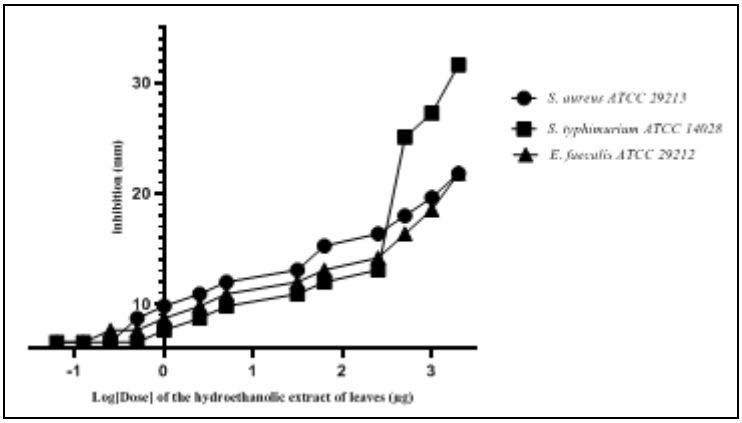

FIG. 6: INHIBITION ZONES OF THE BACTERIA IN FUNCTION OF THE STEM BARK EXTRACT DOSE

Enterococcus faecalis was the most inhibited microorganism with a minimum inhibitory dose of 0.0625 µg/disc, when compared to *Staphylococcus aureus* with a minimum inhibitory dose of 0.250 µg/disc. *Salmonella typhi* was the microorganism that showed the greatest range of inhibition (41.5 - 7.64 mm) at doses 2000 – 0.0625 µg/disc, although it presented the same minimum inhibitory dose as *Enterococcus faecalis* (0.0625 µg/disc). In the experiment with leave extracts, *Staphylococcus aureus* and *Enterococcus faecalis* did not show any variation in inhibition diameter values (21.82 ± 0.00 mm) at maximum concentration (2000 µg/disc). *Salmonella typhi* was the most sensitive microorganism at maximum dose (31.64 ± 0.00 mm), although minimum inhibitory dose for these bacteria was the same as the one obtained with *E. faecalis*, with *E. faecalis* showing a minimum inhibitory dose different from the *Staphylococcus aureus* extract (0.250 µg/disc). The **Fig. 7** shows the correction between the inhibition zone and the concentration of the extract of the leaves.

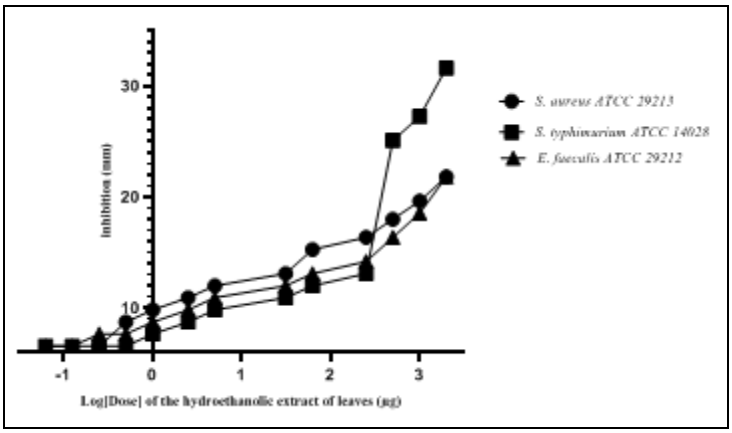

FIG. 7: INHIBITION ZONE OF THE BACTERIA IN FUNCTION OF THE LEAVES DOSE

CONCLUSIONS: The present study demonstrates that all the extracts tested (hydroethanolic extracts from the leaves, stem and stem bark) of *Olax dissitiflora* have in their composition phytoconstituents with therapeutic properties, more specifically the phenolic compounds, and can be used as a good source of polyphenols and alkaloids. Stem was the part of the plant with the maximum value of total phenols and tannins (hydrolysable and condensed) among the studied extracts and otherwise the leaves presented the maximum content of total flavonoids and total alkaloids. The study also demonstrates that aerial parts of *Olax dissitiflora* has a stronger antimicrobial and antioxidant activities. According to the results obtained the stem and the stem bark have the best antibiotic activity with good concentration correlation and the stem has the best antioxidant activity with the lowest EC_{50} values among all the extracts tested, thus suggesting the use of this medicinal species as a source of substances with antioxidant and antimicrobial properties to treat several diseases related with microorganisms and oxidative stress. Furthermore, these results can be used to validate the traditional use of *Olax dissitiflora* in the treatment of skin diseases,

including acne and premature aging, as microbiological and oxidizing agents (free radicals) are implicated in inflammatory and degenerative skin processes.

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Authors' Contributions: Arão Manhique and Carvalho Madivate designed the study and revised. Quitéria Napote collected the data and drafted the manuscript. Paulo Cumbane and Alcides Sitoe analyzed the data and revised the manuscript. All authors approved the final manuscript.

CONFLICT OF INTEREST: The authors have no financial, personal or other relationships with other people or organizations that could inappropriately influence, or perceived to influence their work.

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