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Phytochemical assessment, antiradical and antifungal activity of a recipe composed of three plants: *Khaya senegalensis, Mangifera indica and Ocimum canum*.

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ABSTRACT
Objectives: The present study assessed the pharmacological properties of plants and a recipe used in traditional medicine for the treatment of *Candida albicans* vaginitis in Togo.
Methodology and Results: Qualitative phytochemical analysis was studied using standard tests, Total phenol content, Condensed tannin levels, flavonoid content were determined respectively by the Folin-Ciocalteu method, Butanol-HCl method, and the aluminium chloride method, and free radical scavenging activity by phosphomolybdate reduction and the FRAP method. The microdilution method coupled with spreading on an agar medium was used for the antifungal tests. Phytochemical analysis revealed phenolic compounds, alkaloids, flavonoids, saponosides, tannins, triterpenes, and sterols. *Khaya senegalensis* contains more phenols (97.26±0.10 mg AGE/g) and recorded the best free radical scavenging activity with both methods (2.7±0.00 with the FRAP method and 0.08±0.00 mgAAE/g with the molybdate reduction method (0.08 ±0.00 mg AAE/g). *Mangifera indica* contains more proanthocyanidins (0.98%±0.00 mgCE/g) and more flavonoids (196.35±5.27 mgAGE/g). Antifungal tests showed that all of the plants and that of the recipe had antifungal activity on the germs tested. MICs and MFCs ranged from 3.125 to 50 mg/ml. The recipe extract was fungicidal on most germs tested.
Conclusion and application of results: The results obtained partly justify the traditional use of this recipe in the treatment of *Candida albicans* vaginitis in Togo.
Keywords: *Khaya senegalensis, Mangifera indica, Ocimum canum*, vaginitis, recipe, plants.

INTRODUCTION

Vaginitis is more common after the patient has a vaginal imbalance. Among the various forms of candidiasis, vulvovaginal candidiasis(VVC) is a frequent reason for consultation in gynaecology (Pihet et Marot, 2013, Bergogne-Bérézin, 2007) . Seventy five percent (75%) of women were affected by VVC during their reproductive age. of (Dovnik et *al.,* 2015). Many cases of VVC have an effect on a sexual activity and mental health. (Blostein et *al.,* 2017). *Candida albicans* is usually the pathogen, which is implicated in VVC. Some complications can be observed in CVV including infertility, premature birth, miscarriage and other infectious diseases (Powell et Nyirjesy, 2014) . The high cost of drugs and the phenomenon of antifungal resistance due to the repeated use of antifungals make treatment difficult (Garnaud C, Cornet, 2020) . To find solutions of these problems, new alternatives are needed. New molecules which could be used to fight bacteria could be find in medicinal plants (Mbaveng et *al.*, 2015). Indeed, a review by Alognon et *al.*(2023) on the antifungal activity of medicinal plants in West Africa identified 56 plants studied with 43% of the plants exhibiting good activity *in vitro* (Alognon et *al.*,2023) . Based on an ethnobotanical survey, we focused on *K. senegalensis, M. indica and O. canum*, three plants used to treat vulvovaginal candidiasis in Togo. However, the scientific knowledge available concerning the activity of these plants on microorganisms is insufficient. The overall aim of this study is to contribute to the evaluation of the pharmacological properties of a recipe made up of these three plants used in traditional medicine in Togo for the treatment of vulvovaginal candidiasis.

MATERIALS AND METHODS

Solvents and reagents: methanol, magnesium chloride, iron trichloride, Dragendorff reagent, concentrated sulfuric acid, aluminium chloride, rutin, Folin-Ciocalteu reagent (FCR), gallic acid, potassium bicarbonate, butanol, HCl, 0.6M sulfuric acid, 0.1% sodium phosphate, ascorbic acid, distilled water, ferric chloride, quercetin, chloroform, 95% ethanol, distilled water were purchased from Fisher (USA).

Plant material and extraction: The plant material used consisted of the trunk barks of *K. senegalensis* and *M. indica*, and the whole plant of *O. canum*. Plant samples were collected in Anfoin (Lakes prefecture) and in Vogan (VO prefecture) in the Maritime Region. The plants were shade-dried for two weeks at the laboratory temperature (28±2°C) before being ground into powder. To prepare the hydroethanol extract, 250 g of powder was stirred for 48 h in 3000 ml of 70% dilute ethanol. It was then filtered through Whatman N◦ 1 filter paper. A rotary evaporator was used to evaporated the solvent.The extracts were then frozen and freeze-dried.

Fungal strains: Two reference strains of *C. albicans* (*C. albicans* ATCC 10231 and *C. albicans* ATCC 35659) and 6 strains of *C. albicans* were used as biological material. Strains were provided by the Institut National d'Hygiène.

Qualitative phytochemical testing of extracts: A summary qualitative phytochemical analysis was studied using standard tests Extracts were analysed for phenolic compounds, alkaloids, flavonoids, tannins, triterpenes/sterols, and saponosides (Odeja et *al.*, 2014).

Testing for phenolic compounds: A few drops of iron trichloride ($FeCl_3$) were added to 2 ml of extract. The presence of a blackish-brown coloration showed the presence of phenolic compounds.

Flavonoid detection: Add a few drops of magnesium turnings to 2 ml of extract. When

a few drops of concentrated HCl are added, a yellow-orange or purple-red coloration appears, confirming the presence of flavonoids.

Testing for alkaloids: To 2 ml of extract, one drop of Dragendorff's reagent was added. The presence of alkaloids was confirmed by formation of a precipitate

Test for saponosides: 2 ml of extract in two separate tubes was shaken vigorously for 1 minute. The presence of persistent foam in the extract proves the presence of saponosides. The second tube served as a control.

Triterpenes and sterols: The appearance of a red-brown ring between two phases, one clear at the bottom and the other green (not so much) at the top, after addition of a few drops of chloroform and concentrated sulfuric acid to 2ml of extract indicates the presence of triterpenes and sterols.

Quantitative phytochemical testing of extracts

Polyphenol assay: Total phenol content was assessed using the method described by Singleton et *al.* (1999) taken over by Ouadja et *al.* (2018) . Values were determined by extrapolation on a standard curve, obtained from a series of dilutions of gallic acid (200 mg/l) with distilled water, ranging from 0.1 to 0.25 mg/ml. A mixture of 0.2 ml extract (the sample to be assayed) at 1mg/ml and 0.5 ml FCR diluted 1/2 in distilled water is added to the test tubes. After 5 minutes incubation at room temperature, 0.5 ml sodium carbonate (20 g/l) was added to the mixture. The volume in each tube was made up to 4ml. After shaking, the different solutions are left to stand, protected from light, for 30min. Optical density was read at 760 nm against a negative blank consisting of a mixture of 0.5ml FCR, 0.5ml sodium carbonate and distilled water, and a positive blank consisting of the extract to be determined and distilled water. Three readings are taken per sample.

Proanthocyanidin assay: Proanthocyanidol content was assessed using the Butanol-HCl method, developed by Porter et *al*, (1986) taken over by Ouadja et *al.* (2018). The test consisted in adding to 50 mg of each extract, 0.2ml of ammoniacal iron sulfate (20g/l) and 7ml of a butanol/HCl solution (95/5 ml) in tubes. After 45 min incubation in a water bath at 95°C, absorbances were read at 550 nm. Optical densities were read three times for each extract.

$$X = (OD \times 1CE/g) / 0.280$$

OD = 280 g equivalent to 1% catechin; OD = optical density measured at 540nm; X = equivalent catechin concentration per gram (%CE /g).

Determination of flavonoid content: Flavonoid content was assessed using the method described by Andzi-Barhé et *al.* (2015) . The operation-involved vortexing 1ml of the 1mg/ml extract solution or 1ml of each quercetin concentration with 1ml of 2% aluminium chloride ($AlCl_3$). After 10 min incubation, absorbance will be measured directly with a UV-visible spectrophotometer (METASH UV-5200PC UV/VIS Spectrophotometer) at 415 nm against a blank. Quercetin will be used as a standard. Three tests will be carried out for each extract.

Evaluation of the free radical scavenging activity of the extracts by the phosphomolybdate reduction method: The protocol used is that described by Prieto et *al.* (1999). The phosphomolybdate reagent was prepared (100 ml reagent) from a mixture of 90ml 0.6M sulfuric acid, 5 ml 0.1% sodium phosphate and 5 ml 1% ammonium molybdate. For the test, 1ml of each extract was added to 9ml of the above reagent. The mixture was heated to 95°C in a water bath for 90 minutes, after which it was cooled to room temperature. Optical densities were

measured at 695 nm against a blank consisting of reagent and distilled water. The standard antioxidant used is ascorbic acid, and results were expressed as milligrams of ascorbic acid equivalent per gram of extract noise (mg AAE/g). Tests were carried out in three trials.

Evaluation of antioxidant activity using the FRAP (Ferric Reducing Antioxidant Power) method: The protocol used is that described by Benzie et Strain (1996). For test extract samples, to 3 ml of freshly prepared FRAP assay reagent in a test tube with a mixture of three solutions (acid buffer pH = 3.5 (50 ml), 2,4,6- tripyridyl-s-triazine (TPTZ) solution (5 ml) and iron III chloride solution (5 ml) reagent (3ml), test extract solution (100 µl) of titre 1mg/ml were mixed in the same proportions as for standard curve plotting. Optical density was read after 5 minutes at 593 nm. The antioxidant capacity of the extracts was measured using the calibration curve by the colour change linked to the formation of the complex (Fe2+ TPTZ) and expressed in µmol Eq FeS04/mg dry extract. Trials were repeated 3 times.

Assessment of antifungal activity: In a 96-well plate, three successive series of gradient 2 dilutions were made from the extract stock solutions and MH broth. A series of concentrations of: 100 mg/ml, 50mg/l, 25mg/ml, 12.5 mg/ml. The volume of extract per well is 100 µl. Each well then receives 100 µl of microbial suspension. The test was performed in an aseptic environment under a laminar flow hood (BIO KLONE 2) and repeated three times for each extract *C. albicans* strain. Nystatin Injection 10mg/ml was used as the standard antibiotic. The wells were read after 18-24h incubation in an oven at 37°C. Turbidity and haze deposit in each well were assessed by eye. The Minimum Inhibitory Concentration (MIC) was read, corresponding to the concentration of the well containing the lowest concentration of extract with no visible culture. The Minimum Fungicidal concentration (MFC), which by definition is the lowest concentration that kills 99.99% of the starting inoculum, was determined by spreading a 100 µl aliquot of wells witch concentrations are greater than or equal to the MIC on sabouraud chloramphenicol. Tests were performed three times. Antifungal activity is considered fungicidal if MFC/MIC= 1; fungistatic if MFC/MIC= 2 (Ouadja et *al.*, 2018) .

Study of the synergistic, antagonistic and additive effects of extracts: The effect of combining different extracts was assessed using the method described by Toudji et *al.*, 2018 (Toudji et *al.*, 2018) . Dilutions are always of order 2 for each extract. In each well, 50µl of each dilution of the two extracts to be combined is added to 100µl of bacterial inoculum. The different combinations are assessed by calculating the fractional inhibitory concentration index (FICI). FICI = FICIA + FICIB, where A, B and C are the different extracts. If FICI<1, there is Synergy; 1<FICI<2, No interaction; FICI>2, Antagonism; FICI= 1, Additivity

Statistical analysis: The statistical study was carried out using Graph Pad Prism 5 statistical software and Excel 2016 spreadsheet software. The standard error of the mean accompanied. The difference between means is considered statistically significant at the 5% level ($p<0.05$). Mean values.

RESULTS

Qualitative phytochemical tests: The results of qualitative phytochemical tests are reported in Table 1. They show that the extracts tested contain all the compounds tested, with the exception of *K. Senegalensis*, which contains no alkaloids

Table 1: Results of qualitative phytochemical tests

	phenolic compound	**Alkaloids**	**Flavonoids**	**Saponosides**	**Tannins**	**Triterpenes and sterols**
M. indica	+	+	+	+	+	+
K. senegalensis	+	-	+	+	+	+
O. canum	+	+	+	+	+	+
Recipes	+	+	+	+	+	+

(+: presence; -: absence).

Total phenol, proanthocyanidin and flavonoid contents. Results summarized in table 2 shows that *K. senegalensis* extract is richest in total polyphenols (4.15±0.11 mg GAE/g extract), followed by *M. indica* extract (97.26±0.10 mg GAE/g extract). *O. canum* extract has the lowest total polyphenol value (39.5±0.06 mg GAE/g extract), while *M. indica* extract is richer in proanthocyanidins or condensed tannins (0.98 mg CE/g), while the recipe is richer in flavonoids.

Table 2: Results of quantitative phytochemical tests

Compounds Extracts	**Total polyphenols (mg AAE/g)**	**Proanthocyanidins (mg CE/g)**	**Flavonoids (mg QE/g)**
K. senegalensis	97.26±0.10	0.59%±0.01	177.02±19.39
M. indica	95.68±0.34	0.98%±0.00	196.35±5.27
O. canum	39.5 ± 0.06	0.58%±0.00	93.07±4.80
Recipe	95.25 ± 5.25	0.94%±0.00	213.64±6.14

Anti-free radical activity: The results are summarized in table 3. : Anti-free radical activity showed that *Khaya senegalensis* recorded the best free radical scavenging activity with both methods (2.7±0.00 with the FRAP method and 0.08±0.00 mgAAE/g with the molybdate reduction method. The highest value was obtained with *K. senegalensis* extract (0.08 mg AAE/g of extract).

Table 3: Anti-free radical activity results

Compounds Extracts	**Anti-free radical activity FRAP (mg AAE/g)**	**Anti-free radical activity Phosphomolybdate (mg AAE/g)**
K. senegalensis	2.7±0.00	0.08±0.00
M. indica	2.6±0.00	0.06±0.00
O. canum	2.4±0.04	0.06±0.00
Recipe	2.6±0.00	0.07±0.00

Antifungal activity: The antifungal activity results (Table 4) show that, overall, the four hydroethanolic extracts tested (*K. senegalensis, M. indica, O. canum* and the Three-Plant Recipe) showed antifungal activity on all Candida strains tested at concentrations ranging from 3.125 to 50 mg/ml.

Table 4: Antifungal activity of extracts

		Extracts			
Germs	**Concentrations (mg/ml)**	***K. senegalensis***	***M. indica***	***O. canum***	**Recipe**
	MIC	12.5	12.5	6.25	3.125
C. albicans	**MFC**	25	25	12.5	6.25
ATCC (36591)	**MFC/MIC**	2	2	2	2
	MIC	12.5	12.5	12.5	12.5
C. albicans	**MFC**	25	25	25	25
ATCC(10231)	**MFC/MIC**	2	2	2	2
	MIC	6.25	12.5	6.25	3.125
C. albicans1	**MFC**	12.5	25	12.5	3.125
	MFC/MIC	2	2	2	1
	MIC	12.5	12.5	12.5	6.25
C. albicans	**MFC**	25	12.5	25	6.25
2	**MFC/MIC**	2	1	2	1
	MIC	12.5	12.5	6.25	3.125
C. albicans 3	**MFC**	25	12.5	6.25	3.125
	MFC/MIC	2	1	1	1
	MIC	25	12.5	6.25	12.5
C. albicans 4	**MFC**	25	12.5	12.5	12.5
	MFC/MIC	1	1	2	1
	MIC	6.25	6.25	6.25	3.125
C. albicans 5	**MFC**	6.25	6.25	6.25	3.125
	MFC/MIC	1	1	1	1
	MIC	12.5	25	12.5	6.25
C. albicans 6	**MFC**	25	50	12.5	12.5
	MFC/MIC	2	2	1	2

The lowest MIC (3.125 mg/ml) was obtained with Recipe extracts on *C. albicans* ATCC 36591, *C. albicans* 1, *C. albicans* 3 and *C. albicans 5*. The lowest FMC was obtained with recipe extracts (3.125 mg/ml). *K. senegalensis* bark extract was fungicidal on *C. albicans 4; C. albicans 5;* and fungistatic on all other strains tested. *M. indica* bark extract was fungicidal on *C. albicans 2; C. albicans 3, C. albicans 4; C. albicans 5*, and fungistatic on all other strains. Whole plant extract of *O. canum* was fungicidal against *C. albicans* 3; *C. albicans* 5; *C. albicans* 6, and fungistatic on other strains.

Table 5: Study of synergistic, antagonistic and additive effects of extracts

Combination Strains	*Ks/Mi MIC*	FIC_{Ks}	FIC_{Mi}	*FICI*	*Action*	*Ks/Oc MIC*	*FICKs*	*FICOc*	*FICI*	*Action*
ATCC 36591	6.25	0.5	0.5	1	Ad	6.25	0.5	1	1.5	-
ATCC 10231	6.25	0.5	0.5	1	Ad	6.25	0.5	0.5	1	Ad

Ca1	3.125	0.5	0.25	0.75	S	6.25	0.5	0.5	1	Ad
Ca2	12.5	1	1	2	-	6.25	0.5	0.5	1	Ad
Ca3	6.25	0.5	0.5	1	Ad	6.25	0.5	0.5	1	Ad
Ca4	12.5	0.5	1	1.5	-	25	1	0.5	1	Ad
Ca5	6.25	0.5	1	1.5	-	50	0.5	0.5	1	Ad
Ca6	12.5	0.5	0.5	1	Ad	12.5	0.5	0.5	1	Ad

Ad= additivity; S= synergy; - = no interaction

Table 5 (continued)

Combination Strains	***Mi/Oc MIC***	***FICMi***	***FICOc***	***FICI***	***Action***
ATCC 36591	3.125	0.25	0.5	0.75	S
ATCC 10231	6.25	0.5	0.5	1	Ad
Ca1	6.25	0.5	1	1.5	-
Ca2	6.25	0.5	0.5	1	Ad
Ca3	6.25	0.5	1	1.5	-
Ca4	12.5	1	2	3	An
Ca5	3.125	0.5	0.5	1	Ad
Ca6	6.25	0.25	0.5	0.75	S

Ad= additivity; S= synergy; - = no interaction, Ca = *C. albicans.* The study of the effect of combining extracts showed that 62.5% (10/16) of combinations showed additivity, 12.5% showed synergy and 18.75% showed no interaction and 6.25% showed antagonism.

DISCUSSION

The results show us that the extracts tested all contain flavonoids, triterpenes and sterols, results confirmed by other authors (Pritesh et Zara, 2015 ; Fagbohoun, 2014 ; Takin *et al*., 2014 ; Atto *et al.*, 2016 ; Ononamadu *et al*., 2019) . Alkaloids are present in all extracts tested except in *K. senegalensis* extracts. Fagbohoun confirmed the absence of alkaloids in *K. senegalensis* in 2014, while Fagbohoun (2014) and Harbone et *al.* (2000) proved the presence of alkaloids in *M. indica* and *O. canum*. Several studies have been carried out on *K. senegalensis, M. indica and O. canum*. However, quantitative phytochemical studies of phenolic compounds in extracts from these plants are limited. In the present study, *M.indica* recorded a proanthocyanidin content of 0.98%±0.00 mgCE/g. This result is at odds with the work carried out by Dweck (2005), who showed that mango bark contains 16-20% condensed tannins. These differences could be explained by the variation of methodology, pedological and climatic differences. The phenolic compound content found would explain the antifungal activity of the extract of (*K. senegalensis, M.indica and O. canum*), as would the recipe. As phenols have the ability to inhibit the germination of pathogenic plant spores, they are excellent candidates for combating pathogenic fungi in humans (Cushnie et Lamb, 2005). Other experimentations demonstrated that phenolic compounds have antioxidant properties (Koudoro et *al.*, 2014) . In addition, the free radical scavenging activity of these plant extracts and the recipe was determined by the phosphomolybdate reduction and by the FRAP method. These directly measure the total reducing capacity of the antioxidant present in the samples (Phatak et Hendre, 2013). The free radical scavenging activity of reducing compounds measured in this way is quantified in milligram ascorbic acid equivalent per gram (mg EAA/g) of extract. A good correlation was observed between the

proanthocyanidin content of the extracts and the results of free radical scavenging activity (r^2 = 0.94). Studies by Ouadja et *al.*(2018) found the same correlation (Ouadja et *al.*, 2018) . The highest value was obtained with *K. senegalensis* extract (0.08 mg EAA/g extract).These results corroborate the work of Martinez, et *al.* (2009) and Androulakis et *al.* (2006) and the lowest value was obtained with *M. indica* and *O. canum* extracts (0.06 mg ascorbic acid equivalent per gram extract). These results confirm those obtained by Martinez, et *al.* (2009) and Makut et *al.* *(*2008) who demonstrated the antiradical activity of *K. senegalensis* trunk bark extracts. These results confirm that *K. senegalensis* is a potential source of antioxidants. These results confirm use of this recipe in medicine. The antifungal power of *K.senegalensis* extract was studied against 8 strains of Candida. Our results confirm those of Makut et *al.*(2008) who found in 2008 that *K. senegalensis* bark extract has a fungicidal effect against *C. albicans*. Earlier work demonstrated the antifungal efficacy of *K. senegalensis* bark extract *in vitro* on three vegetable fungal pathogens in the Sokoto metropolis at different concentrations 1mg/ml; 3 mg/ml; 6 mg/ml; 9 mg/ml and 12mg/ml. These extracts were found to be effective in reducing the growth of these pathogenic fungi to a percentage inhibition of (83.05±0.53%) (Shehu et *al.*, 2016.) The antifungal activity of *M. indica* extract was evaluated on 8 Candida strains. Results show the inhibitory properties of *M. indica* extract on Candida strains, with MICs ranging from 6.25 to 50 mg /ml. These results concur with those of Disegha GC, Akani (2017) who found that the ethanolic extract of *M. indica* proved sufficiently active against *C. albicans* with a MIC of 20 mg/ml. The antifungal activity of *O. canum* extract was studied against 8 Candida strains. Our results concur with those obtained by Thaweboon S, Thaweboon (2009) who demonstrated the antifungal activity of *O. canum in vitro* against *C. albicans*. The extract of the recipe composed of the three plants: *K. senegalensis*; *M. indica* and *O. canum* were fungicidal on germs tested. This fungicidal power is due to the additive effect of the three plants in the recipe on the germs.

CONCLUSIONS AND APPLICATIONS

This work is part of the evaluation of the activity of three plants and a traditional recipe based on three plants, *K. senegalensis*, *M. indica* and *O. canum* used in the treatment of vulvovaginal candidiasis in Togo. The results confirm that the recipe has antifungal activity. Its action was fungicidal on most of the Candida strains tested; with a Minimum Fungicidal Concentration ranging from 3.125 to 50mg/ml. Phytochemical analysis reveals the presence of several chemical groups. Determination of total polyphenols showed their presence in large quantities in the hydroethanolic extracts of *K. senegalensis*, *M. indica* and the recipe exerts antiradical activity. The results obtained partly justify the traditional use of this recipe in the treatment of vulvo-vaginal candidiasis, and merit further research.

AUTHOR CONTRIBUTIONS

Conceptualization, A.A. and B.D.; methodology, A.A., S.P., A.G.T. software, S.P., A.A.; formal analysis, A.A., S.P.; resources, M.E.S.M., B.D.; data curation, S.P., A.A.; writing—original draft preparation, A.A, S.P., M.E.S.M., Y.H., L.G.; writing—review and editing, A.A., S.P., E.H.G., Y.H.; visualization, S.P., L.G., Y.H.,

M.E.S.M. B.D., All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare that they have no competing interests.

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