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Phytochemical Screening, Secondary Metabolites Assay, Antioxidant, Antibacterial and Antifungal Activity of Clove Buds (*Syzygiumaromaticum*) Extracts

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Abstract: ***Clove buds (Syzygiumaromaticum) are one of the most widespread plants and their essential oil is by far the most widely used part. In this study, which first focused on the phytochemical characterization of aqueous, methanolic, and acetone extracts, the methanolic one is the richest and contains all the secondary metabolites sought, with the exception of leucoanthocyanins and emodins. Then, an assay of alkaloids, flavonoids, and polyphenols is done by colorimetric methods. The results showed that aqueous infused is richer in alkaloids, followed by methanolic extract with concentrations of 6.71 µg/mg and 2.55 µg/mg respectively. For the flavonoid composition, organic solvent extracts are the richest, namely acetone extract with a concentration of 132.66 µgQE/mg, followed by methanolic extract at 140.84 µgQE/mg and the quantification of polyphenols has also shown that methanolic extract is richer, followed by infused extract with concentrations of 438 and 414.5 µgAGE/mg. In addition, the study of antioxidant activity by the ABTS method showed that infused is the most active extract with an IC50 of 1.6 µg/ml lower than that of ascorbic acid used as standard. And finally, for the study of antimicrobial activity, extracts are more active against Staphylococcus aureus, against which MICs of 5.7 µg/ml are obtained with the aqueous fraction of the infused and 11.5 µg/ml with the ethyl acetate fraction. Against Candida albican the smallest MIC is obtained with the ethyl acetate fraction which is equal to that of Neomycin B sulfate and is 187 µg/ml.***

Keywords: Clove dubs, alkaloids, polyphenols, antibacterial, antifungal, antioxidant

1. Introduction

Syzygium aromaticum is a plant native to the Moluccas in Indonesia. It is cultivated for its fruits, and clove buds, harvested and marketed for their therapeutic properties.

In Senegal, *Syzygium aromaticum* is not an indigenous plant; cloves are generally imported from Brazil. These parts of the plant are widely used for their antibacterial or antifungal effects but also in combination with tea for its taste or other herbal teas for their aphrodisiac properties [1].

Many studies have been done on the essential oil of *Syzygium aromaticum* which has shown antibacterial [2]; [3], antifungal [4] antioxidant [5].

However, these imported and marketed cloves have not been the subject of local scientific studies to validate this extensive use.

The objective of this study is to perform phytochemical characterization, and quantify secondary metabolites in order to study the antioxidant, and antimicrobial activities of aqueous and organic extracts of cloves of *Syzygium aromaticum*.

2. Materials and Methods

2.1 Plant material

The plant material consists of clove buds powder of *Syzygiumaromaticum*.

2.2 Extraction and fractionation

Test portions of 20 g of clove bud powder were infused with 200 ml of water at 90 °C until cooling. The mixture were filtered and the infused were concentrated to dryness on a rotary evaporator. These dry extracts were used for the tests and fractionation. A sample of 2 g of the dry extract obtained by infusion were dissolved in 100 ml of water and

then subjected to fractionation with ethyl acetate and chloroform successively. The ethyl acetate, chloroform, and aqueous fractions were evaporated to dryness, and the residues obtained were used for the various tests.Two test samples of 10 g of clove bud powder were separately macerated in a volume of 100 ml of acetone and 100 ml of methanol respectively, for 1 day. Then the blend obtained in each case were filtered before being dry-concentrated with a rotary evaporator.

2.3 Phytochemical Screening

Preliminary qualitative phytochemical screening were carried out with the following methods.

Steriods: 1 ml of the extract were dissolved in 10 ml ofchloroform and equal volume of concentrated sulphuricacid were added by sides of the test tube. The upper layerturns red and sulphuric acid layer showed yellow withgreen fluorescence. This indicated the presence ofsteroids [6].

Terpenoids: 2 ml of extract were added to 2 ml of aceticanhydride and concentration of H_2SO_4. Formation of blue,green rings indicate the presence of terpenoids [7].

Fatty Acids: 0.5 ml of extract were mixed with 5 ml of ether.These extract was allow it for evaporation on filter paperand dried the filter paper. The appearance of transparenceon filter paper indicates the presence of fatty acids [7].

Tannins: 2 ml of extract were added to few drops of 1%lead acetate. A yellowish precipitate indicated thepresence of tannins [8].

Saponins: 5 ml of extract were mixed with 20 ml of distilledwater and then agitated in a graduated cylinder for 15minutes. Formation of foam indicates the presence of saponins [9].

Anthocyanins: 2 ml of aqueous extract were added to 2 ml of 2N HCl and ammonia. The appearance ofpink-red turns blue-violet indicates the presence of anthocyanins [10].

Leucoanthocyanins: 5 ml of aqueous extract addedto 5 ml of isoamyl alcohol. Upper layer appearsred in colour indicates for presence of leucoanthocyanins [10].

Coumarins: 3 ml of 10% NaOH were added to 2 ml ofaqueous extract formation of yellow colour indicates the presence of coumarins[11].

Emodins: 2 ml of NH OH and 3 ml of Benzene were addedto the extract. Appearance of red colour indicates the presence of emodins[11].

2.4 Dosage of total alkaloids

The alkaloids were dosed according to the method described by Sreevidya. A test sample of 5 ml of 5 mg/ml extract solution of previously prepared concentration in a pH aqueous solution maintained at 2-2.5 with diluted HCl and then a 2 ml quantity of Dragendorff (DR) reagent a were added and the precipitate formed was centrifuged. The centrifugate were checked for complete precipitation by adding DR. After centrifugation, the centrifugate was decanted completely and meticulously. The precipitate were further washed with alcohol. The filtrate were discarded and the residue was then treatedwith a 2 mL disodium sulfide solution.

The brownish-black precipitate formed were then centrifuged. Completion of precipitation were checked by adding 2 drops of disodium sulfide. The residue were dissolved in 2 mL of concentrated nitric acid, with warming if necessary. This solution were diluted to 10mL in a standard flask with distilled water; 1 mL were then pipetted out, and 5 mL thiourea solution was added to it. The absorbance were measured at 435 nm against the blank containing nitric acid and thiourea. A calibration curve were carried out in parallel under the same operating conditions using bismuth nitrate. The amount of bismuth present in the solution were calculatedby multiplying the absorbance values with the factor, taking the suitable dilution factor into consideration [12].

2.5 Dosage of total flavonoids

The total flavonoid content of each plant extract were determined by a colorimetric method. 0.5 ml of the aqueous extract solution at 1 mg/ml concentration were mixed with 2 ml of distilled water and subsequently with 0.15 ml of a $NaNO_2$ solution (15%). After 6 min, 0.15 ml of aluminium chloride ($AlCl_3$) solution (10%) were added and allowed to stand for 6 min, then 2 ml of NaOH solution (4%) were added to the mixture. Immediately, water were added to bring the final volume to 5 ml and the mixture were thoroughly mixed and allowed to stand for another 15 min. The absorbance of the mixture were then determined at 510 nm versus the prepared water blank. Results were expressed as quercetin equivalent (µg quercetin/mg dried extract) [13].

2.6 Dosage of total polyphenols

The total polyphenols content of each plant extract were determined with the Folin–Ciocalteu's reagent (FCR) according to the published method [14]. 0.1 ml of aqueous extract solution at 1 mg/ml concentration were mixed with 2 ml of a freshly prepared 2% Na_2CO_3, the whole were stirred by a vortex. After five minutes, 100 µl of the Folin-Ciocalteu reagent (1 N) were added to the mixture, the whole were left for 30 minutes of incubation at room temperature and the reading were performed against a blank. The absorbance were then measured at 700 nm. Results were expressed as gallic acid equivalent (µg gallic acid/mg dried extract).

2.7 Experimental protocol of the ABTS test

A quantity of 38.40 mg of ABTS were dissolved beforehand in 10 ml of water.A quantity of 6.75 mg of potassium persulfate were added thereafter. The mixture obtained were kept in the dark and at ambient temperature for 12 hours before use. This mixture werediluted with ethanol in order to obtain an absorbance of the order of 0.7 to 734 nm.The

antioxidant activity were measured by mixing 0.8 ml of the extract dissolved in ethanol with 3.2 ml of the ABTS solution in order to obtain concentrations varying from 1 to 10 µg/ml. Ascorbic acid, used as reference antioxidants, were dissolved in ethanol and tested at the same concentrations. The absorbance reading were made after 2 minutes at the spectrophotometer at 734 nm using ethanol as blank. Three absorbance measurements were performed for each concentration [13].The antioxidant activity were denominated as the percentage inhibition (PI) of the absorbance of the radical which corresponds to:

$$\mathbf{PI} = \frac{\mathbf{A0} - \mathbf{A1}}{\mathbf{A0}} \mathbf{x100}$$

A0: absorbance of the solution of ABTS
A1: absorbance of the solution of ABTS after addition of the extract tested to a given concentration and after the reaction.
IC_{50} values are calculated from GraphPad Prism software (v5.0d, San Diego, CA) using a non-linear regression model using percent inhibition (PI) values.

2.8 Preparation of extract solutions

The various extracts and fractions obtained were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 6 mg/ml.

2.9 Antibiotics standard

The reference antibiotics used are amoxicillin (United States Pharmacopoeia standard lot N° L0K369), cloxacillin sodium (European Pharmacopoeia lot N° 3a), and neomycin B sulfate (International Chemical Reference Substance Control N° 193178). Each of these substances were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 6 mg/ml.

2.10. Antimicrobial tests

Microorganismes
The microbial strains used in this study were obtained from the Bacteriology and Virology Laboratory of the Aristide Le Dantec hospital at Dakar. Those studied consist of reference strains: *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 32219, and *Pseudomonas aeruginosa* ATCC 29853, *Candida albican*. The bacterial species were planted in a nutrient medium and incubated at 37 °C for 24h.

Culture media
The culture media used in this study are Mueller-Hinton agar and broth (MH) for bacterial tests and Sabouraud agar and broth for fungal tests.

Determination of antibacterial activity
The disc diffusion methods were used to test the antimicrobial activities of different extracts. In this light, Petri dishes containing Mueller Hinton medium (MH) were seeded with inoculum to 10^5CFU/ml in a swabbed system. Sterile discs of 0.5 cm in diameter were placed on the surface of solid culture media contained in Petri dishes and already seeded with bacterial or fungal inoculum at 10^5CFU/ml by swabbing. Then a volume of 20 µl of extract solution and antibiotic standard previously dissolved in DMSO at a concentration of 6mg/ml were deposited on each disc. The dishes were introduced into an oven at 37 °C for 24h.The antimicrobial activity were determined by measuring the diameter of the zone of inhibition around the discs.

Determination of the minimum inhibitory concentration (MIC)
Determining the MICwere performed as described below.

With microplates containing 100µl of broth per well, dilution series ranging from 3mg/ml to 6 µg/ml were performed with the extract and antibiotic standard solutions used to perform the activity tests.Then each well 10µL bacterial culture suspension at 10^5 UFC were filed.

A clear staining of the well were interpreted as the lack of growth and wells showing a cloudy appearance were considered positive because of the growth of bacteria.

3. Results

Clove buds extracts composition on secondary metabolites of medicinal plants used to treat different ailments

Table 1: Results of phytochemical characterisation of the extracts

Secondary metabolites	Infused	MeOH	Acetone
Steriods	+	+	+
Terpenoids	-	+	+
FattyAcids	+	+	+
Tannins	+	+	+
Saponins	+	+	-
Anthocyanins	-	+	+
Leucoanthocyanins	+	-	-
Coumarins	-	+	+
Emodins	+	-	-

Note: + = indicates presence and - = absence
Infused = infusion extract, MeOH= methanol extract, Acetone= acetone extract

Results of dosage tests of total alkaloids, total flavonoids and total polyphenols
The following table shows the results of secondary metabolite testing in plant samples.

Table 2: Quantities of alkaloids, flavonoids and polyphenols µg/mg of plant extracts

Extracts	Total alcaloids [µg/mg]	Total flavonoids [µgQE/mg]	Total polyphenol [µgAGE/mg]
Infused	6,71	94,48	414,5
EA.F	5,6	170,38	748
C.F	6,11	141,8	159,5
W.F	4,32	118,38	387
MeOH	2,55	132,66	438
Acetone	2,23	140,84	395,5

Note: Infused = infusion extract, MeOH= methanol extract, Acetone= acetone extract, EA.F= faction of ethyl acetate, C.F= chloroform fraction, WF= water fraction

Results of the antioxidant test of extracts by ABTS method

Table 3: Percentage of inhibition of the extracts and fractions of the extract by infusion of leaves tested with ABTS

	Extract and fraction					
[µg/ml]	Infused	EA.F	C.F	W.F	MeOH	Acetone
2 µg/ml	42,76	24,33	33,01	30,14	39,47	29,03
4 µg/ml	78,03	50,15	56,07	47,81	76,68	70,77
6 µg/ml	79,77	67,33	77,46	65,68	94,84	75,11
8 µg/ml	99,57	77,18	80,42	77,74	98,80	85,64
10 µg/ml	100	94,52	94,36	91,06	99,73	90,12
IC_{50}	1,63	3,07	2,50	2,86	2,10	2,32

The CI_{50} of the ascorbic acid used as a reference is 1.83 µg/ml.
Note: Infused = infusion extract, MeOH= methanol extract, Acetone= acetone extract, EA.F= faction of ethyl acetate, C.F= chloroform fraction, WF= water fraction

Results of the antimicrobial essay of the extracts and the antibiotic standard

Table 4: Inhibition diameter of the extracts and antibiotic standards around the discs perminimeter (mm) at 6mg/ml of concentration

	Souches microbiennes			
Extract and antibiotic	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Candida albican*
Infused	12	7	8	8
EA.F	16	6	7	9
C.F	7	-	-	6
W.F	10	-	-	6
MeOH	16	8	7	11
Acetone	10	9	8	8
Amoxicillin	42	32	-	22
Cloxacillin	44	14	-	16
Neomycin B sulfate	25	21	6	17

Note: Infused = infusion extract, MeOH= methanol extract, Acetone= acetone extract, EA.F= faction of ethyl acetate, C.F= chloroform fraction, WF= water fraction,- = Not active,

The numbers represent the inhibition diameters around the disc per mm.

Table 5: Values of minimum inhibitory concentrations of the extract µg/ml

	Souches microbiennes			
Extract and antibiotic	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Candida albican*
Infused	23	750	750	750
EA.F	11.5	281	375	187
C.F	46	750	187	375
W.F	5.7	750	187	750
MeOH	11.5	750	750	750
Acetone	46	187	750	750
Amoxicillin	<1.5	3	375	750
Cloxacillin	<1.5	187	187	750
Neomycin B sulfate	93	<1.5	141	187

Note: Infused = infusion extract, MeOH= methanol extract, Acetone= acetone extract, EA.F= faction of ethyl acetate, C.F= chloroform fraction, WF= water fraction, the numbers represent the MIC values of each extract or antibiotic per µg/ml, < =less than.

4. Discussion

Phytochemical characterization makes it possible to highlight the composition of secondary metabolites in plant extracts. Many studies have shown that these secondary metabolites are responsible for the therapeutic effects observed after the use of plants.

Indeed, phytochemical characterization has shown that the richest extract is methanolic because it contains all the compounds sought except leucoanthocyanins and emodins. These three extracts are also complementary because the phytochemical compounds absent in one extract are found in the others (Table 1). Given this rich composition of extracts, it would be important to do a secondary metabolite assay. For the quantification of secondary metabolites performed by colorimetric assay, the infused is the richest in alkaloids, followed by methanolic extract with concentrations of 6.71 µg/mg and 2.55 µg/mg respectively. This can be explained by the nature of the alkaloids, which would be more soluble in water than in organic solvents. These alkaloids migrate largely after fractionation into the chloroform phase where the concentration is 6.11µg/mg (Table 2).For the flavonoid composition, organic solvent extracts are the richest, namely acetone extract with a concentration of 132.66 µgQE/mg, followed by methanol extract at 140.84 µgQE/mg. After fractionation, these flavonoids present in the extract by aqueous infusion migrate mostly to the ethyl and aqueous acetate fraction (Table II). The quantification of polyphenols has also shown that methanolic extract is richer followed by infusion with concentrations of 438 and 414.5 µgAGE/mg. But in the partitioning of the infused, polyphenols are mainly concentrated in the ethylacetate phase where the concentration is 748 µgAGE/mg. This also provides information on the polarity of these polyphenols (Table 2). These phytochemical compounds are responsible for observed biological effects such as antioxidants.

The study of antioxid ant activity by the ABTS method showed that infused is the most active extract with an IC_{50} of 1.6 µg/ml lower than that of ascorbic acid used as standard. It is followed by methanolic extract which has an IC_{50} of

2.10µg/ml and acetone with an IC_{50} of 2.32 µg/ml. With the fractions of the infused, the antioxidant power decreased on all fractions (Table 3). These results also show that flavonoids and phenolicacids are the most important groups of secondary metabolites and bioactive compounds in plants [15].The antioxidant power of the infusion also shows that water-soluble alkaloids would be good antioxidants (Table 2 and Table 3). These compounds may also have antibiotic activities. For the test of antibacterial and antifungal activities, the extracts are more active against Staphylococcus aureus which is a Gram-negative bacterium, against which MICs of 5.7 µg/ml are obtained with the aqueous fraction of the infused and 11.5 µg/ml with the ethylacetate fraction. Under the same conditions, the fractions were more active than the reference antibiotic used, Neomycin B sulfate. With Gram-negative bacteria, the lowest MIC (187 µg/ml) is obtained with acetone extract against Escherichia coli. This MIC is equal to that of Cloxacillin against the same strain. A MIC of 187 µg/ml is obtained against Pseudomonas aeruginosa with chloroformic and aqueous fractions, however, amoxicillin at a MIC of 375 µg/ml. Staphylococcus aureus is both a commensal bacterium and a human pathogen. (Table 4, Table 5).This pendant, approximately 30% of the human population is colonized with Staphylococcus aureus [16] and one of the most frequent causative pathogens of all different types of osteomyelitisis Staphylococcus aureus [17] and Methicillin-resistant Staphylococcus aureus (MRSA) features prominently in these epidemics. Historically associated with hospitals and other health care settings, MRSA has now emerged as a widespread cause of community infections [18]. Against Candida albican, the smallest MIC is obtained with the ethylacetate fraction which is equal to that of Neomycin B sulfate and is 187 µg/ml. These results are obtained in a context where Candida albican can lead to serious disease that is fatal in 42% of cases [19].

5. Conclusion

These results show that cloves are a plant rich in secondary metabolites. This richness makes the plant an important source of antioxidants and antibiotics, especially with the infusion. The interesting results of this study may explain the frequency of the use of these cloves by the Senegalese population, which is probably satisfied with the fact that the aqueous extract has shown more activity.

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