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Antimicrobial Activities of Extracts of Tobacco Leaf (*Nicotiana tabacum*) and Its Grounded Snuff (Utaba) on *Candida albicans* and *Streptococcus pyogenes*

Anumudu CK[1*], Nwachukwu MI[2], Obasi CC[3], Nwachukwu IO[2] and Ihenetu FC[2]

[1]*Department of Microbiology, Federal University Otuoke, Bayelsa State, Nigeria*

[2]*Department of Microbiology, Imo State University Owerri, Nigeria*

[3]*Department of Public Health, Imo State University Owerri, Nigeria*

[*]**Corresponding author:** Anumudu CK, Department of Microbiology, Federal University Otuoke, Bayelsa State, Nigeria, Tel: 08034457946; E-mail: anumuduck@fuotuoke.edu.ng

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Abstract

This study was designed to investigate the antimicrobial activity of leaf and ground snuff extracts of Tobacco (*Nicotiana tabacum*) against *Candida albicans* and *Streptococcus pyogenes* using methanol and water as extracting solvents. The study employed the agar diffusion and tube dilution assays. Methanol extracts of tobacco leaf produced zones of inhibition of 13.0 mm against *Streptococcus* and 9.5 mm against *Candida*, whereas the water extracts produced inhibition zones of 10.0 mm for *Streptococcus* and no inhibitory activity on *Candida*. A minimum inhibitory concentration of 25 mg/ml was recorded by the methanol extracts of tobacco leaves against *Candida* and 100 mg/ml MIC against *Streptococcus*. The methanolic leaf extracts had both bactericidal and fungicidal effect on both *Streptococcus* and *Candida* at a concentration of 200 mg/ml. The zones of inhibition obtained from methanolic extracts of grounded snuff against *Streptococcus* was 10.5 mm and 15.0 mm against *Candida* whereas the water extracts produced inhibition zones of 7.5 mm for *Streptococcus* and 11.0 mm against *Candida*. A minimum inhibitory concentration of 100 mg/ml was recorded by both methanolic and *water* snuff extracts against *Streptococcus*. While the MIC obtained from the methanolic extract of ground snuff against Candida was 50 mg/ml. The water extracts of ground snuff showed no bactericidal or fungicidal activity. Whereas 200 mg/ml of the methanolic extract of ground snuff was microbiocidal against *Streptococcus* and *Candida*. In summary, the study showed that Grounded snuff is more of an antifungal agent than antibacterial while tobacco leaves have great antibacterial potential. This may justify the use of tobacco leaves and its ground snuff in the treatment of oral thrush caused by *Candida albicans* and strep throat caused by *Streptococcus pyogenes*.

Keywords: Antimicrobial activity; Methanol extract; Water extract; Tobacco; Ground snuff

Introduction

Innumerable biologically active compounds that are found in plants possess antibacterial properties [1,2]. Plant-produced compounds are of interest as sources of safer or more effective substitutes for synthetically produced antimicrobial agents [3]. Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world. The World Health Organization estimates that plant extracts or their active constituents are used as folk medicine in traditional therapies of 80% of the world's population [4]. The antibacterial activities of these leaves are due to the presence of various secondary metabolites [5].

In recent years, secondary plant metabolites (phytochemicals), previously with unknown pharmacological activities, have been extensively investigated as sources of medicinal agents [6]. Thus, it is anticipated that phytochemicals with adequate antibacterial and antifungal efficacy will be used for the treatment of bacterial and fungal infections. Since time immemorial, man has used various parts of plants in the treatment and prevention of various ailments [7]. Tobacco (*Nicotiana tabacum*) is an herbaceous perennial plant, native to tropical and subtropical America and cultivated worldwide [8]. Extracts of tobacco have been shown to effectively inhibit various Gram-positive, Gram-negative bacteria and acid-fast *Mycobacterium phlei* [9]. It was also effective against the opportunistic *Candida albicans* and *Cryptococcus neoformans* [10]. Furthermore, *in vivo* studies by Russel et al., [9] showed that high concentrations of nicotine in tobacco users reduced microbial loads in the oral cavity of smokeless tobacco users. Yildirim et al., [11] demonstrated that ether and ethanol extracts of seeds and leaves of tobacco had antimicrobial activities on *Staphylococcus*. While Wang et al., [5] demonstrated antimicrobial activities of tobacco extracts on *E. coli, Staphylococcus aureus* and *Bacillus subtilis*. The aim of this study is to evaluate the activity of extracts from Tobacco leaf and its ground snuff against *Streptococcus pyogenes* and *Candida albicans in vitro*.

Material and Methods

Collection of samples

Tobacco leaf and ground snuff used for this research were obtained from local retailers of herbs at Ekeonunwa market in Owerri Imo state Nigeria and identified at the Department of Plant Science and Biotechnology, Imo State University Owerri. The collected leaves were washed thoroughly with tap water followed with sterile distilled water for the removal of dust and soil particles. The leaves were shade dried

for few days until they attained a steady weight and then homogenized into a finely powdered form and stored in an airtight jar.

Cultures used

Test micro-organisms (*Streptococcus pyogenes* and *Candida albicans*) used for this study were obtained from stock cultures of the Federal Medical Center Owerri, Imo State. The viability of the test organisms was confirmed by growing on Nutrient Agar and Sabouraud Dextrose Agar respectively. *Streptococcus spp.* was re-identified using Gram staining and biochemical tests namely; indole, urea, citrate, catalase and coagulase tests, following the scheme of Cheesbrough [12]. Confirmation of *Candida albicans* was by microscopy and Germ Tube Test as described by Alexopolous et al. [13].

Stock cultures were maintained at 4°C on slopes of nutrient agar and Sabouraud Dextrose Agar for *Streptococcus pyogenes* and *Candida albicans* respectively. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of Mueller-Hinton Broth (MHB), these are incubated without agitation for 24 hrs at 37°C. The cultures were diluted with fresh Mueller-Hinton to achieve optical densities of 2.0×10^6 cfu/ml (MacFarlands standard).

Preparation of plant extracts

50 grams of the homogenized leaf samples and 50 grams of the grounded snuff were extracted with 500 ml of methanol using soxhlet apparatus at 80℃. Further, the solvent was evaporated using a rotary vacuum evaporator. The residue was dissolved with Dimethyl Sulfoxide (DMSO) and used for the antimicrobial activity.

Antimicrobial activity

Agar diffusion assay: The disc diffusion method was used to screen the antimicrobial activity. In vitro antimicrobial activity was screened by using Mueller Hinton Agar (MHA). The MHA plates were prepared by pouring 15 ml of molten media into sterile Petri-plates. The plates were allowed to solidify for 5 minutes and 0.1 ml inoculum suspension was swabbed uniformly and the inoculum was allowed to dry for 5 minutes. Different concentrations of extracts were loaded on 6 mm sterile disc. The loaded disc was placed on the surface of the medium and allowed to diffuse for 5 minutes before incubation at 37°C for 24 hrs. At the end of incubation, inhibition zones formed around the disc were measured with a transparent meter rule. Each assay was performed in duplicates and mean values were utilized.

Tube dilution assay (MIC test): The MIC test is carried out to determine the lowest concentration of the extract that is able to inhibit the growth of the test organism. This test is performed using Muller Hinton broth. For each of the extracts (methanol and water), for grounded snuff and tobacco leaf, 5 ml of Muller Hinton broth is placed in seven test tubes. 1ml of the extract is introduced into the first tube and serial dilution of the extract is undertaken to reduce the concentration of the extract in the broth serially.

Next, a standard inoculate (0.1 ml) of the test organism (adjusted to the MacFarlands standard) is introduced into each of 5 test tubes. The remaining two test tubes served as controls. In one control, the broth was inoculated with the test organism while the crude extract was added to the other. All the tubes were incubated overnight in an incubator at 37°C.

After overnight incubation, the broths were examined for turbidity. Turbidity resulting from microbial growth indicates that the extract concentration present in the test tube had no antimicrobial effect on the test organism. Tubes that were clear mean that there was no microbial growth. The MIC is the broth containing the lowest concentration of the extract, which showed no growth (was able to inhibit microbial growth).

Minimum microbiocidal concentration test: This test is performed to determine amongst the tubes/concentrations that showed growth inhibition, which of them actually was able to kill all the micro-organisms present. This test is performed by inoculating a sterile nutrient agar plate with a loopful obtained from the nutrient broth tubes that showed no growth (clear). The Petri-dishes were incubated overnight in an incubator maintained at a temperature of 37°C and observed for the formation of colonies.

If the concentration of the extracts in the Muller Hinton broth tube was able to only inhibit the growth of the microbes, without affecting cell death, there would be growth on the Petri-dishes. However, if the concentrations were able to kill the cells, colonies would not be formed on the agar plates. The MBC is the lowest concentration of the extract that caused cell death.

Results

In the disc diffusion assay for grounded snuff extracts, the extracts gave varied zones of inhibitions, with the highest been recorded by the methanol extracts against *Candida albicans* with a zone diameter of 15.0 mm. The largest zone diameter recorded against *Streptococcus pyogenes* was also by the methanol extracts with a zone diameter of 10.5 mm (Table 1).

Streptococcus pyogenes			*Candida albicans*		
Plant extract	**Concentration (mg/ml)**	**Zone of inhibition (mm)**	**Plant extract**	**Concentration (mg/ml)**	**Zone of inhibition (mm)**
Methanol	200	10.5	Methanol	200	15
	100	8.5		100	14
	50	6		50	12.5
	25	6		25	11
	12.5	6		12.5	6

Water	200	7.5	Water	200	11
	100	7		100	11
	50	6		50	9
	25	6		25	8.5
	12.5	6		12.5	6

Table 1: Agar diffusion assay of different extracts of grounded snuff.

On analysis of the inhibitory properties of the various extracts using the tube dilution assay, only the concentrations of 200 mg/ml and 100 mg/ml of methanol and water extracts respectively were able to inhibit the growth of *Streptococcus pyogenes*. Whereas *Candida albicans* was readily inhibited, with a MIC of 50 mg/ml and 100 mg/ml for the methanol and water extracts, respectively (Table 2).

Streptococcus pyogenes			*Candida albicans*		
Plant extract	**Concentration (mg/ml)**	**Turbidity**	**Plant extract**	**Concentration (mg/ml)**	**Turbidity**
Methanol	200	Clear	Methanol	200	Clear
	100	Clear		100	Clear
	50	Turbid (+)		50	Clear
	25	Turbid (++)		25	Turbid (+)
	12.5	Turbid (++)		12.5	Turbid (++)
		MIC=100 mg/ml			MIC=50 mg/ml
Water	200	Clear	Water	200	Clear
	100	Clear		100	Clear
	50	Turbid (++)		50	Turbid (+)
	25	Turbid (++)		25	Turbid (++)
	12.5	Turbid (++)		12.5	Turbid (++)
		MIC=100mg/ml			MIC=100mg/ml
KEY=(+) Slight turbidity (++) Heavy turbidity					

Table 2: Tube dilution assay (MIC) of different extracts of grounded snuff.

On sub-culturing, the broth cultures that showed growth inhibition unto nutrient agar, only the methanol extract of 200 mg/ml had a bactericidal effect against *Streptococcus pyogenes* and fungicidal activity against *Candida albicans* (Table 3).

Streptococcus pyogenes			*Candida albicans*		
Plant extract	**Concentration (mg/ml)**	**Colony formation**	**Plant extract**	**Concentration (mg/ml)**	**Colony formation**
Methanol	200	No growth	Methanol	200	No growth
	100	growth		100	Growth
	50	Δ		50	Growth
	25	Δ		25	Δ
	12.5	Δ		12.5	Δ

		MBC=200 mg/ml			MBC=200 mg/ml
Water	200	Growth	Water	200	Growth
	100	Growth		100	Growth
	50	Δ		50	Δ
	25	Δ		25	Δ
	12.5	Δ		12.5	Δ
		NO MBC			NO MBC
KEY (Δ)=no MBC test was carried out for this concentration, as this concentration showed no antimicrobial activity in the tube dilution (MIC) test.					

Table 3: Minimal microbiocidal concentration test of different extracts of grounded snuff.

In the disc diffusion assay for tobacco leaf extracts, the extracts gave varied zones of inhibitions, with the highest been recorded by the methanol extracts against *Streptococcus pyogenes* with a zone diameter of 13.0 mm. The largest zone diameter recorded against *Candida albicans* was also by the methanol extracts with a zone diameter of 9.5 mm (Table 4).

Streptococcus pyogenes			*Candida albicans*		
Plant extract	**Concentration (mg/ml)**	**Zone of inhibition (mm)**	**Plant extract**	**Concentration (mg/ml)**	**Zone of inhibition (mm)**
Methanol	200	13	Methanol	200	9.5
	100	11		100	8.5
	50	6		50	7.5
	25	6		25	6
	12.5	6		12.5	6
Water	200	10	Water	200	6
	100	9		100	6
	50	6		50	6
	25	6		25	6
	12.5	6		12.5	6

Table 4: Agar diffusion assay of different extracts of tobacco leaf.

On analysis of the inhibitory properties of the various extracts using the tube dilution assay, the methanol extract concentrations of 100 mg/ml and 25 mg/ml was able to inhibit the growth of *Streptococcus pyogenes* and *Candida albicans*, respectively. The water extracts were able to inhibit microbial growth with a MIC of 200 mg/ml and 100 mg/ml for *Streptococcus pyogenes* and *Candida albicans* respectively (Table 5).

Streptococcus pyogenes			*Candida albicans*		
Plant extract	**Concentration (mg/ml)**	**Turbidity**	**Plant extract**	**Concentration (mg/ml)**	**Turbidity**
Methanol	200	Clear	Methanol	200	Clear
	100	Clear		100	Clear
	50	Turbid (+)		50	Clear
	25	Turbid (++)		25	Clear
	12.5	Turbid (++)		12.5	Turbid (++)

		MIC=100mg/ml			MIC=25mg/ml
Water	200	Clear	Water	200	Clear
	100	Turbid (+)		100	Clear
	50	Turbid (++)		50	Turbid (+)
	25	Turbid (++)		25	Turbid (++)
	12.5	Turbid (++)		12.5	Turbid (++)
		MIC=200 mg/ml			MIC=100 mg/ml
Key=(+) Slight turbidity (++) Heavy turbidity					

Table 5: Tube dilution assay (mic) of different extracts of tobacco leaf.

On sub-culturing, the broth cultures that showed growth inhibition unto nutrient agar, only the methanol extract of 200 mg/ml had a bactericidal and fungicidal effect against *Streptococcus pyogenes* and *Candida albicans*. The water extracts had no microbiocidal effect on the test organisms (Table 6).

Streptococcus pyogenes			*Candida albicans*		
Plant extract	**Concentration (mg/ml)**	**Colony formation**	**Plant extract**	**Concentration (mg/ml)**	**Turbidity**
Methanol	200	No growth	Methanol	200	No growth
	100	growth		100	Growth
	50	Δ		50	Growth
	25	Δ		25	Growth
	12.5	Δ		12.5	Δ
		MBC=200 mg/ml			MBC=200 mg/ml
Water	200	Growth	Water	200	Growth
	100	Δ		100	Growth
	50	Δ		50	Δ
	25	Δ		25	Δ
	12.5	Δ		12.5	Δ
		NO MBC			NO MBC
Key (Δ)=no MBC test was carried out for this concentration, as this concentration showed no antimicrobial activity in the tube dilution (MIC) test.					

Table 6: Minimal microbiocidal concentration test of different extracts of tobacco leaf.

Discussions

Snuff extract showed varied antimicrobial activities against *Streptococcus pyogenes* in the agar diffusion assay. The largest zones of inhibition were obtained from the methanol extracts, with a zone diameter of 10.5 mm. On carrying out the tube dilution test, only the higher concentrations of 200 mg/ml and 100 mg/ml of the different extracts were able to inhibit the growth of the bacteria, while the lower concentrations had no inhibitory effect. When the tubes that showed inhibition (clear) were subcultured unto Nutrient agar plate, only the methanol extract of 200 mg/ml showed a bactericidal effect. This is similar to the result obtained by Yildirim et al., [11] on *Staphylococcus aureus* and by Wang et al., [5] on *E. coli*. The water extract showed only inhibitory effects against *Streptococcus pyogenes*. Similarly, the snuff extracts had antifungal activity against *Candida albicans*. The agar diffusion assay recorded large zones of inhibition for the methanol extracts with the largest zone of inhibition recorded as 15.0 mm. The tube dilution assay recorded a Minimum Inhibitory Concentration (MIC) of 50 mg/ml for the ethanol extract and 100 mg/ml for the water extract. When the tubes that showed growth inhibition were sub-cultured unto Nutrient agar plates, only the methanol extract (200 mg/ml) had a fungicidal effect. Other extracts were only able to inhibit but not kill the fungi.

Extracts obtained from tobacco leaves had an antibacterial effect on *Streptococcus pyogenes* and hence recorded a wide zone of inhibition against the test organism in the agar diffusion assay. The largest zone of inhibition was obtained from the methanol extracts with a zone diameter of 13.0 mm. On carrying out tube dilution assay, a Minimum Inhibitory Concentration (MIC) of 100 mg/ml was obtained from methanol extract while water extract recorded a MIC of 200 mg/ml. The minimum microbiocidal concentration test showed that only methanol extract (concentration=200 mg/ml) had bactericidal activity. This is similar to the result obtained by Okorondu et al., [14] on *Staphylococcus aureus* and *Escherichia coli*. On the fungal assay, tobacco leaf extract showed a little antifungal effect against *Candida albicans*. In the agar diffusion assay, only the methanol extract showed a significant zone of inhibition (9.5 mm). On carrying out the MIC test, the methanol extract recorded the lowest MIC of 25 mg/ml against *Candida albicans*. Only the methanol extract of 200 mg/ml had a fungicidal effect.

Overall, grounded snuff showed the greatest activity against *Candida albicans* (*in vitro*) based on the agar diffusion test, while the tobacco leaf extracts showed greater activity against *Streptococcus pyogenes*.

Conclusion

Tobacco has a long history of being used as a remedy for many ailments. Analysis of the various results obtained shows that tobacco leaf extracts and those of grounded snuff truly have antimicrobial properties as proposed by traditional medical practitioners. Grounded snuff is more of an antifungal agent than antibacterial while tobacco leaves have great antibacterial potential. It is possible that these natural extracts would have lower human toxicity and hence can be used as microbial growth inhibitors of both *Candida* and *Streptococcus* infections. The antimicrobial properties of tobacco leaves and ground snuff are already being exploited by traditional medical practitioners in the treatment of various ailments. It is therefore recommended that further research should be carried out to determine the active phytochemical components and purify them for use as novel antimicrobial drugs. Also, the toxicity associated with the use of these extracts should be tested *in-vivo* before it can be used on humans for the treatment of *Candida* and *Streptococcal* infections.

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