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Full Length Research Paper

In vitro activity of *Thaumatococcus daniellii* and *Megaphrynium macrostachyum* against spoilage fungi of white bread and 'Eba', an indigenous staple food in Southern Nigeria

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Fresh white bread and 'eba' (a carbohydrate; > 80% starch, indigenous staple food in southern Nigeria) were purchased from traders in Lagos, Nigeria and stored under aseptic conditions in the laboratory at room temperature (27 ± 2°C) (11 h of darkness and 15 h of daylight) for duration of 144 h, during which samples were analyzed at 48 h intervals for fungal population using the plate count technique. The sensitivity of the spoilage fungi to aqueous and methanol extracts of leaves of *Thaumatococcus danielli* and *Megaphrynium macrostachyum* was determined using the broth dilution method. Fungal populations increased with hour of storage. For bread, *Penicillium* sp was dominant throughout with average populations of 2.0 x 10^1 and 8.01 x 10^6 cfu/g at 0 and 144 h, respectively, while *Mucor* sp was dominant in eba with average populations of 1.0 x 10^1 and 9.5 x 10^6 cfu/g at 0 and 96 h, respectively. For *T. danielli*, while the methanol extract had greater activity against the spoilage fungi than the aqueous extract, the reverse was observed for *M. macrostachyum*. The lowest minimum inhibitory concentration (MIC) of *T. danielli* was 25 mg/ml of the methanol extract against *Saccharomyces cerevisiae* and *Saccharomyces chevalieri* and its lowest minimum cidal concentration (MCC) was 50 mg/ml of the methanol extract against *S. cerevisiae* and *Penicillium* sp. For *M. macrostachyum*, the lowest MIC and MCC were 25 and 50 mg/ml of the aqueous extract respectively against *S. chevalieri*. The results highlight the potential of extracts of *T. daniellii* and *M. macrostachyum* as sources of alternative natural preservatives of bread and 'eba'.

Key words: *Thaumatococcus danielli*, *Megaphrynium macrostachyum*, minimum inhibitory concentration, minimum cidal concentration.

INTRODUCTION

Microbial spoilage of bread decreases the shelf life of bread and may result in substantial economic losses to the producers (Sorokulova et al., 2003) and potential health hazards to consumers. Baking is expected to destroy microorganisms that may be present in dough and as such make the freshly baked bread sterile. It has, however, been reported that bread is prone to contamination by mould spores and bacteria from non sterile equipment and the air during cooling and before wrapping (Pepe et al., 2003; Sorokulova et al., 2003) and that mould spores in proofers' cloths in bakeries can become heat resistant to survive baking (Ogundare and Adetuyi, 2003, Sorokulova et al., 2003). Eba, a carbohydrate [composed of >80% starch and about 1% protein (Ahonkai and Koleoso, 1979; Sanni et al., 2002)] food indigenous to southern Nigeria, prepared from gari (roasted fermented cassava; *Manihot esculenta* Crantz, flour) using hot water (at 100°C) may be contaminated and spoilt by microorganisms surviving the preparation process and those introduced by handlers.

The problem of food spoilage is surprisingly assuming

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greater dimension, especially in the developing countries (Walker, 1994) . This could be due to several factors, one of which is the current legislative and consumer pressure to reduce use of preservatives, particularly, the organic acids derived ones, in intermediate moisture bakery products like bread, and in food generally (Lavermicocca et al., 2003). Both manufacturers and consumers now desire fresher, addictive-free, more natural tasting food products while not compromising microbiological safety and stability (Olasupo et al., 2003). Applying the EU directive, many European countries have stipulated the reduction of the levels of chemical preservatives in bakery products. But these reduced levels, such as 0.3% (wt/vol) calcium propionate, have been reported to be ineffective (Lavermicocca et al., 2003) . Also, this action is likely to stimulate mould growth, since such preservatives are fungistatic and not fungicidal (Keshri et al., 2002). Here lies the background for the increased interest in biopreservation of food system which has led to the development of new natural antimicrobial compounds of animal, plant and microbial origins (Lavermicocca et al., 2003, Olasupo et al., 2003, Al-Turki et al., 2008).

Thaumatococcus danielli Benn. (Benth) (Family: *Marantaceae*) is indigenous to the rainforest of West Africa (Arowosoge and Popoola, 2006: Ojekale et al., 2007). The fruit contains 1 to 3 black seeds surrounded by a gel and capped with a membranous sac; the aril contains the sweet protein called thaumatin. In addition to being the source of the natural sweetener: thaumatin in food and confectionary industry, the leaves are used for wrapping and boiling food in Nigeria and Ghana as a way of extending the shelf life of the food (Ojekale et al., 2007). *T. danielli* has contributed to the rural economy for a long time but, it's potential has not been fully exploited at the industrial level.

Megaphrynium macrostachyum (K.Schum.) (Family: *Marantaceae*) is found in the rainforest of West and Central Africa (Jennings et al., 2001). The leaves are harvested from the forest and used fresh in wrapping food in order to preserve the food.

T. danielli and *M. macrostachyum* were selected for this study in order to justify their local use in preservation of food and to explore their potentials as sources of alternative natural preservatives of bread and eba.

MATERIALS AND METHODS

Isolation and measurement of microbial population

Fresh white bread and 'eba' were bought from road side traders at Egbeda and Ojo (between latitude 4°15/ North and Lat 4°17/ North and longitude 12°55/ East and 13° East) areas of Lagos State, Nigeria. One gram each was separately weighed from the food samples and homogenized with 9 ml of sterile distilled water, using sterilized blender. From each of these initial dilutions, serial dilution was carried out up to the 10^{-4} dilutions. From the 10^{-4} dilutions, 1 ml was aseptically transferred onto the surface of Sabouraud Dextrose Agar (SDA) plates in duplicates. The plates were incubated at 25°C for 24 – 48 h (11 h of darkness and 15 h of daylight). After incubation, the microbial counts were recorded and distinct colonies were subcultured for isolation of pure cultures. The process was repeated at intervals of 48 h till 144 h. The isolates were identified according to standard microbiological procedures (Klich and Pitt, 1988; Pitt, 1988; Samson et al., 1995).

Collection and preparation of plant materials

Fresh leaves of *T. danielli* and *M. macrostachyum* were bought from the Mile 12 market in Lagos and were immediately transported to the laboratory in polythene bags. The leaves were authenticated at the Botany Department of Lagos State University, Ojo. The leaves of the two plants were washed separately with distilled water and dried at 60°C for 24 h in the oven. The leaves were pulverized using sterile pestle and mortal, after which they were ground into fine powder using the Hammer mill (scotmic model). The fine powder was accordingly weighed and labeled.

Aqueous extraction of plant leaves

Weighed powdered leaves (200 g) of *T. danielli* and *M. macrostachyum* were separately added to 2 L distilled water in conical flasks and the mouth covered with sterile aluminium foil. The flasks were left for 4 days at room temperature and carefully but vigorously shaken at intervals. The resultant mixtures were filtered in membrane filters and the extracts concentrated using a rotary vacuum evaporator. The extracts were freeze–dried in pre-weighed sterile bottles and stored at 4°C in the refrigerator until needed.

Methanolic extraction of plant leaves

Weighed powdered leaves (80 g) of *T. danielli* and *M. macrostachyum* were added to fractionating column after which 1000 ml of methanol was used to run through the soxhlet extraction process for 8 h. The methanol was recovered by distillation after which the extract were freeze–dried in pre–weighed sterile bottles and stored at 4°C in the refrigerator until needed.

Determination of minimum inhibitory concentrations

Four grams of each extract were separately added to 10 ml distilled water as diluent (to have the 400 mg/ml) and serial 2- fold dilutions were made. Each dilution was inoculated with a drop (0.02 ml) of the logarithmic phase culture of each of the mould isolates in Sabouraud dextrose broth (SDB) diluted to 0.5 Macfarlane standard. Sabouraud dextrose broth inoculated with the isolates without plant extract and SDB with plant extract without microorganism were set up as controls. The plates were incubated at 25°C (11 h of darkness and 15 h of daylight) for 48 h. The lowest concentration showing no visible growth was recorded as the minimum inhibitory concentration (MIC) for each organism (Adeniyi et al., 2000).

Determination of minimum fungicidal concentrations

From each negative tube in the MIC assay, 1 ml was transferred onto the surface of freshly prepared SDA plates and the plates were incubated at 25°C for 48 h. The lowest concentration showing no visible growth on SDA was recorded as the minimum fungicidal concentration (MFC) for each organism (Adeniyi et al., 2000).

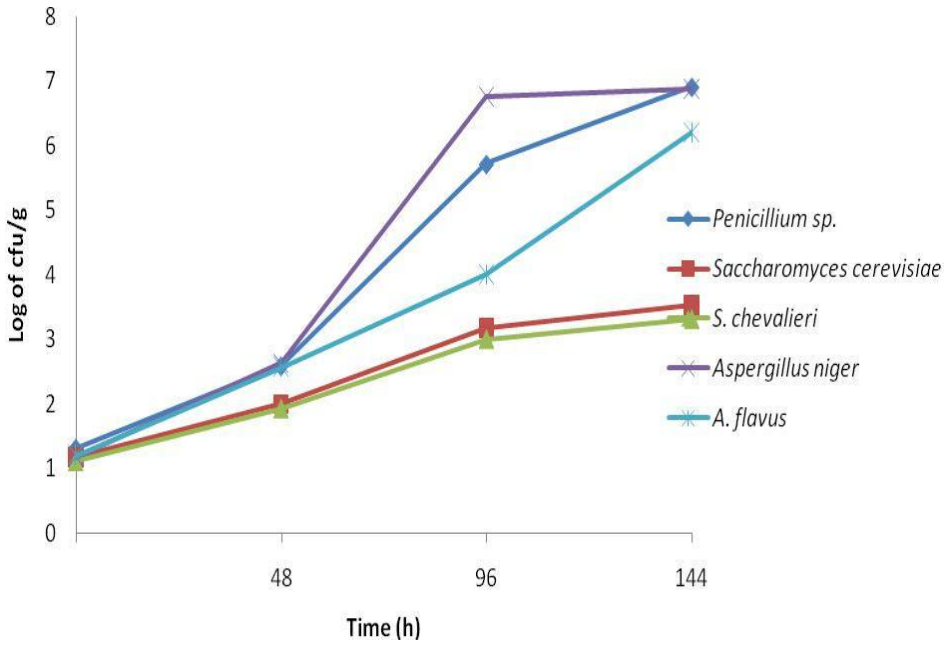

Figure 1. Population of spoilage fungi of bread over a 144 h storage period.

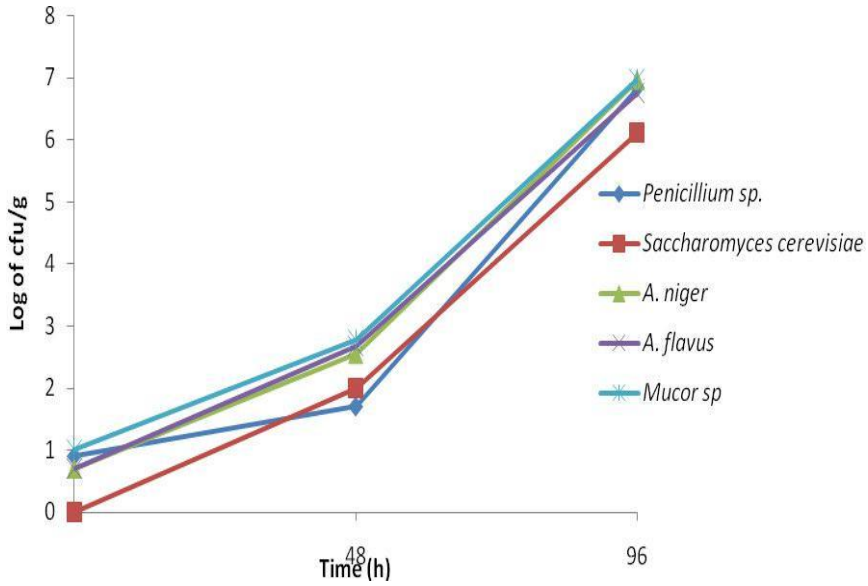

Figure 2. Population of spoilage fungi of eba over a 96 h storage period.

RESULTS

Figures 1 and 2 show that commensal spoilage fungi: *Saccharomyces cerevisiae*, *S. chevalieri*, *Rhizopus, Mucor* and *Penicillium* species constituted majority of the isolates, which also included two pathogenic spoilage fungi: *Aspergillus niger* and *Aspergillus flavus*. Also, the fungal populations for both bread and eba increased with time throughout the storage period and while the fungal populations at 0 h were greater in bread than eba, the rate of proliferation was higher in eba than in bread such that the greatest fungal population in eba was 9.5 x 10^6 cfu/g at 96 h while that of bread was 8.01 x 10^6 cfu/g at 144 h. The *in vitro* activity assay results in Table 1 revealed that the methanol extract of *T. danielli* had greater activity against the test organisms than its aqueous extract. Its lowest MIC was 25 mg/ml of its methanol extract (against *S. cerevisiae* and *S. chevalieri*)

Table 1. Minimum inhibitory and minimum cidal concentrations of extracts of *T. danielli* against spoilage fungi of bread and 'eba'.

Fungal isolates	Minimum inhibitory and minimum cidal concentrations (mg/ml)	
	Aqueous extract	Methanol extract
Penicillium sp.	100 (200)	50 (50)
S. cerevisiae	100 (200)	25 (50)
S. chevalieri	200 (400)	25 (100)
A. niger	400 (-)	400 (-)
Rhizopus sp	50 (200)	50 (200)
A. flavus	400 (-)	400 (-)
Mucor sp.	100 (200)	100 (200)

Minimum cidal concentration in parenthesis.

Table 2. Minimum inhibitory and minimum cidal concentrations of extracts of *M. microstachyum* against spoilage fungi of bread and 'eba'.

Fungal isolates	Minimum inhibitory and minimum cidal concentrations (mg/ml)	
	Aqueous extract	Methanol extract
Penicillium sp.	50 (100)	100 (100)
S. cerevisiae	100 (100)	100 (100)
S. chevalieri	25 (50)	50 (100)
A. niger	200 (-)	100 (200)
Rhizopus sp	50 (100)	400 (-)
A. flavus	200 (400)	200 (400)
Mucor sp.	100 (200)	200 (400)

Key: Minimum cidal concentration in parenthesis.

and the lowest MIC of its aqueous extract was 50 mg/ml (against *Rhizopus* sp.). In Table 2, the results showed that the aqueous extract of *M. macrostachyum* was more active against the test organisms than its methanol extract. Its lowest MIC was 25 mg/ml of its aqueous extract (against *S. chevalieri*) and the lowest MIC of its methanol extract was 50 mg/ml.

DISCUSSION

Some fungi isolated from the bread and eba samples in this study: *S. cerevisiae, S. chevalieri, Rhizopus* and *Mucor* sp were common bread moulds and commensals. However, the presence of *A. niger* , *A. flavus* and *Penicillium* sp. among the isolates might constitute health hazards, since these organisms are known to produce mycotoxins (Bacha et al., 1988). Lavermicocca et al. (2003) reported that potentially toxigenic *Aspergillus ochraceus, A. flavus, Penicillium roqueforti, Penicillium verrucosum* and *Penicillium citrinum* have been previously found in bakery products: moldy bread and rye bread. Microbial growth on bread and eba was easily detected at about 96 h by the development of colour, odour and roppiness signifying spoilage. Roppiness is a bacterial spoilage of bread that initially occurs as an unpleasant fruity odour, followed by enzymatic degradation of the breadcrumb that turns soft and sticky due to the production of extracellular slimy polysaccharides and eventually form visible web–like strands (Thompson et al., 1993). It is instructive; however, that some of the isolates were spore formers and the possibility of consuming the spores even before their germination to vegetative cells that may indicate spoilage cannot be ignored. The work of Keshri et al. (2002) is significant in early detection of contaminating or spoilage microorganisms. They reported the detection of early phases of contamination by *Penicillium chrysogenum* and *Eurotium* sp. prior to visible growth on a bread substrate by their volatile production patterns using an electronic nose or odour mapping system.

The fungal population in the bread and eba samples increased throughout the storage period. This was probably due to the fact that bread and eba become drier with storage time, thereby favouring fungal proliferation. Ogundare and Adetuyi (2003) reported similar observation with bread and attributed it to the dehydration occurring in the bread due to loss of moisture to the environment. The fungal populations reported by Ogundare and Adetuyi (2003) were less than those reported in this study, probably due to the fact that they studied bread baked in a university bakery and directly

transported to the laboratory for analysis. This present work, however, studied bread bought from road side traders. Previous workers have identified sources of microbial contaminants in bread to include: contaminated flour, poorly sterilized equipment, unsanitary baking environment, handlers and exposure of bread to street dusts before wrapping (Pepe et al., 2003; Sorokulova et al., 2003 and Ogundare and Adetuyi, 2003). Likely sources of microbial contaminants in eba include contaminated garri (roasted cassava flour), undue exposure and handlers.

The initial microbial load (microbial load at 0 h) was higher in bread than in eba for all the isolates. This is probably due to the greater number of sources of contaminants in bread than eba, since eba has not been developed into an industrial product. The rate of fungal proliferation was higher in eba than in bread such that at 96 h, the greatest fungal population recorded for eba was higher than the greatest recorded for bread even at 144 h. Also, eba became completely spoilt and discarded after 96 h while bread only became completely spoilt and discarded after 144 h. This was probably due to the presence of some preservative in the bread and the lack of preservative in eba.

The *in vitro* antifungal activity assay results revealed that for *T. danielli*, the methanol extract had greater activity against the fungal isolates than the aqueous extract. For *M. macrostachyum,* the aqueous extract had better performance than the methanolic extract. It is probable that the bioactive compounds in *T. danielli* were more extractable in methanol than in water, while those of *M. macrostachyum* were more extractable in water than in methanol. Egwari (1999) stated that the activity of plant extract can be influenced by the dissolubility of the bioactive compounds in the extractive solvent. Apart from the extractive solvent, another factor that may influence the activity of a plant extract is the method of extraction. Ojekale et al. (2007) observed variation in activity against *Candida albicans* and *A. flavus* by ethanolic and methanolic extracts of *T. danielli* extracted by soxhlet and steeping methods. In this study, both test plant extracts had relatively similar antifungal activity against the test organisms. While there is dearth of literature on the antimicrobial compounds and activity of *M. macrostachyum*, it has been reported that *T. danielli* produces a thaumatin-like protein that is resistant to pH and heat induced denaturation (Ogata et al., 1992), and has antifungal activities against several fungi *in vitro*, inducing the rupture of fungal hyphae at the growing tip (Huynh et al., 1992; Ojekale et al., 2007).

Conclusion

This study indicated the efficacy of the test plant extracts in controlling the growth of the spoilage fungi and, therefore, highlighted their potentials as sources of alternative natural preservatives in the food industry. However, studies on the shelf life of bread and eba preserved with extracts of these plants separately and in combination, along with toxicology screening may be useful in determining the possible application of these extracts in the preservation of food.

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