Aspects of *Garcina kola* Toxicity to a Wild Strain of *Saccharomyces cerevisiae* from a Brewery Environment

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Abstract

Bitter cola (Garcinia kola) powder and its ethanolic extract were assessed for their ability to inhibit the growth of a wild strain of Saccharomyces cerevisiae isolated from a brewery environment. The iso-alpha acid contents of the G. kola powder, G. kola extract and hop extract were determined as 5.85 mg/l, 6.34 mg/l and 7.65 mg/l, respectively, showing that the iso-alpha acid contents of G. kola extract and G. kola powder compared relatively well with that of hop extract. The antimicrobial activity of both agents, using Humulus lupulus ethanolic extract as control, showed a high dependence on the temperature of assay. At most of the temperatures evaluated (30 – 45 ^{o}C), the rates of antimicrobial activity differed. Garcinia kola ethanolic extract and hops extract displayed total inhibition of the wild yeast growth at temperature of 40 ^{o}C at concentrations of about 0.039 mg/ml and 0.078 mg/ml respectively. Humulus lupulus extract (hops) had the highest generation time at 45^{0}C at a concentration of 0.078 mg/ml. The complete inhibition of the yeast growth by G. kola extract at 40 ^{o}C indicated that it had higher antimicrobial activity against this yeast than hops extract. In all the temperatures evaluated both G. kola ethanolic extract and powder compared favourably with hops extract with respect to antimicrobial activity against a wild strain of Saccharomyces cerevisiae at various concentrations. These results revealed that the antimicrobial activity of these hopping agents were temperature and concentration dependent.

Keywords: *Garcinia kola*, Hops, Ethanol extract, Powder, Temperature, Antimicrobial activity

Introduction

Hops are produced from the flowers of the plant known as *Humulus lupulus* and are widely used in beer production. They are responsible for the bitterness, flavor, colour, foam head stability and antimicrobial properties in beer (Hough, 1986). However, hop plant is a temperate crop and attempts to grow it in tropical countries like Nigeria have failed. In 1985, it cost Nigeria about 5.5 million dollars to import hops for beer brewing (Federal office of Statistics (1986). This high cost could be reduced if hops substitutes can be sourced locally (Okoro *et al.*, 2007). Hops are bitter and contain α-acids, iso-α-acid and other essential oils. The bitterness and antimicrobial action of hops in beer are due to the presence of phenolic compounds (Aina and Uko, 1991). A number of some locally available vegetables with bittering properties have been investigated as possible substitutes for hops. They include bitter leaf (*Gongronema latifolium*), utazi leaf (*Vernonia amygdalina*), bitter cola (*Garcinia kola*) (Gentalium, 1975; Okafor and Anichie, 1983; Anichie and Uwakwe, 1990; Aina and Uko, 1991, Iwu and Okolo, 1993; Okoro *et al.*, 2007). The trial of these tropical hop substitutes were based on their high contents of α-acids, iso-α-acid and essential oils which were comparable to those of the temperate hop product (Okafor and Anichie, 1983; Okoro, 1993).

Garcina kola (family, Guttiferae), is commonly known as bitter cola and it is a tropical tree which is cultivated mostly in the southern parts of Nigeria and some other parts of West and Central Africa for its edible fruits and seeds. The tree bears male and female flowers separately. Bitter cola (*Garcinia kola*) seeds are smooth, elliptically shaped, with yellow pulp and brown seed coat (Eleyinmi *et al.*, 2006 and Akerele *et al.*, 2008). The seeds of *G. kola* are used widely in traditional African Medicine for the treatment of a number of diseases due to the activity of their flavonoids as well as other bioactive compounds (Okunji and Iwu, 1991; Okunji *et al.*, 2002; Farombi, 2003; Adegboye *et al.*, 2008). In addition, pharmacological studies have established the presence of a wide range of active compounds in *G. kola* seeds, all of which have important pharmacological relevance (Monago and Akhidue, 2002; Akerelere *et al.* 2008).

The potential utilization of *Garcinia kola* in brewing operations has been reported (Aniche and Uwakwe, 1990; Dosunmu and Johnson, 1995; Ogu and Agu, 1995; Eleyinmi and Oloyo, 2001; Eleyinmi *et al*., 2004). The current considerations of *G. kola* as replacement for hops stems from the fact that they contain α- and iso-α- acids as well as other flavonoid compounds which are responsible for the bittering and antimicrobial effects of hop (Bishop, 1968). In addition, *G. kola* belongs to the same family as hops (*Humulus lupulus*) and preliminary investigations have shown it to have a wide range of antimicrobial activity against some microorganisms. There are scanty reports on the antimicrobial effects of *Garcinia kola* extracts on beer spoilage microorganisms (Ogu and Agu, 1995; Aina and Uko 1991; Aniche and Uwakwe 1990). *G. kola* and hop extracts exert similar antimicrobial effects on beer spoilage microorganisms. Moreso, Akerelere *et al.* (2008) reported the antimicrobial activity of ethanol extracts and fractions of seeds of *G. kola* against *Staphylococcus aureus, Bacillus subtilis*, *Escherichia coli*, *Streptococcus viridians*,

Pseudomonas aeroginosa, *Klebsiella pneumonia*, *Aspergillus niger*, *Penicillium notatum* and *Candida albicans*. The present study examined the antimicrobial activity of the ethanolic extract and powder of *G. kola* seeds against a wild yeast strain of the *Saccharomyces cerevisiae* isolated from a brewery environment. The study forms part of a systematic approach in developing *G. kola* as a viable local alternative for the replacement of *H. lupulus*.

Materials and Methods

Saccharomyces cerevisiae wild yeast strain from a brewery environment was used for this study. The yeast strain was obtained from Dr. C.I. Ezeogu of the Department of Microbiology, University of Nigeria, Nsukka. The organism was inoculated from agar slants into 50 mls of malt extract broth. The malt extract broth was prepared by adding 1g of malt extract into 50 ml of deionized water. The culture was then incubated in a shaker for 12 – 18 hours at room temperature. The cells, after 18h, were aseptically transferred to sterile centrifuge tubes and centrifuged for 20 minutes (10,000 rpm). The supernatant was drained while the cells were harvested and washed three times in sterile Ringers solution.

Preparation of *G. kola* powder: *G. kola* seeds were procured fresh from Nsukka main market. They were washed, decorticated, sorted and dried at 50 ^{o}C for 24h to a moisture content of 10 ± 2% in drought air oven. Thereafter they were milled into powder, using hammer mill (Chrysty – Lab Mill model 8) to 0.1 m diameter particle size.

Preparation of *G. kola* extract: The ethanol extraction of the active ingredients of the *G. kola* was carried out using the method of Ezeifika *et al.* (2004) with some modifications. Hundred grams (100g) of ground *G. kola* seed was soxhlet extracted with 400 ml of 95% ethanol over 24 h. The extract was then vacuum evaporated to a thick brown paste.

Extraction for determination of iso-α-acid content of the hopping agents: This extraction was done to determine the level of α-acid and iso-α-acid in the *G. kola* powder and the *H. lupulus* hop extract. To three bottles each containing 20 ml of synthetic wort, 0.2g each of *G. kola extract*, *G. kola* powder and hops were added respectively. These were boiled for 14 h and thereafter centrifuged for 20 minutes at 10,000 rpm and the supernatant was collected. A 0.4 ml of the supernatant of each of the boiled extracts [*G. kola* extract (GKE), *G. kola* powder (GKP) and hops] was added to a separate centrifuge tube. Three millilitre of iso-octane (85%) was then added. The tube was shaken vigorously for 3 minutes. The emulsion formed was then separated into layers by centrifuging at 10,000 rpm. 1 ml of the upper layer in each of the 3 tubes was mixed with 4 ml of alkaline methanol. A blank was also prepared consisting 1 ml of iso-octane and 4 ml of alkaline methanol.

Thereafter the absorbance of each of the three hopping agents was determined using a spectrophotometer (Pye-Unicam SP6-550 uv/vis. Model) at 255 nm and the iso-α-acid (mg/L) was calculated according AOAC (2000) method of analysis using the formula: Iso-α-acid (mg/L) = A255 (96.15) + 0.4

α-Acid determination: α-acid was determined by the method adopted by Okoro and Aina (2007). To 0.15 g of each of the hopping agents was added 100 ml of cold methanol in a Gallenkamp flask shaker. The solution was thereafter centrifuged at 2500 rpm for 20 min and the decanted supernatant was acidified with 0.002 N HCl. The respective absorbance at 325 nm, 355 nm and 275 nm was determined using a spectrophotometer (Pye-Unicam SP6-550 uv/vis. Model). The α-acid content was calculated according to the methods of AOAC (2000) and ASBC (1976) using the formula: α-Acid (mg/L) = 73.79 (A325) – 51.56 (A355) – 19.07 (A275), where A is absorbance reading at the specified wavelength.

Preparation of synthetic wort: The synthetic wort was produced by dissolving glucose 12g, maltose 66g, fructose 33g, sucrose 40g, dextrin 25g, casamino acids 3.5 g, peptone 3 g, $CaCl_2$ 1.0 g, $MgS0_4$ 1.0 g, KCl 1.0 g, H_3BO_4 10 µg, $ZnS0_4$ 10µg, $MaCl_2$ 10µg, $FeSO_4$ 5 µg, $CuS0_4$ 1.0 µg, KI 1.0µg, inositol 10 mg, calcium pantothenate 1.0 mg, nicotinic acid 1.0 mg, pyridoxine 1.0 mg, biotin 0.5 mg, guanosine 0.5 mg, uracil 0.5 mg, thiamine 1.0 mg and bovine serum albumin 1.0 mg in one litre of distilled water. The mixture was then sterilized by autoclaving at 121^{0}C for 15 minutes and thereafter used as synthetic wort.

Antimicrobial assay of extracts: Sample of each hopping agent was prepared by weighing 0.2g of the agent and boiling it in 20 ml of synthetic wort for 1h. Following boiling, the sample was clarified by centrifugation at 10, 000 rpm for 15 min. The clear supernatant was subsequently sterilized by autoclaving. A 2-fold serial dilution was performed through the serial transfer of tubes containing 3 ml sterile synthetic wort.

Effect of hopping agents (*G. kola* powder, *G. kola* ethanolic extract and hop extract) on yeast growth: The synthetic wort with the appropriate dilution of the hopping agent was inoculated with 0.05 ml of *Saccharomyces cerevisiae* yeast suspension (OD of 0.1 units at 600 nm). The effect of the hopping agent on the yeast growth rate was monitored at 2 hourly intervals for 16 h by taking O.D. reading at 600 nm. The effects of the hopping agents against the wild yeast were evaluated at different temperatures (30 ^{o}C – 45 ^{o}C) and concentrations. The generation time of the yeast was calculated by the following equation: G = 693/µ = generation time, where µ = specific growth rate = ln {(OD_1 / OD_0) / t} where OD_1 = optical density after incubation, OD_o = initial optical density and t = time in minutes between initial reading and final reading.

Results and Discussion

The iso-α-acid contents of *G. kola* powder, *G. kola* extract and hop extract were 5.85 mg/L, 6.34 mg/L, and 7.65 mg/L, respectively (Table 1).

Table 1: The α-acid and iso-α-acid content of the three hopping agents

Hopping agents	Iso-α-acid (mg/L)	α-acid (mg/L)
Hop extract	7.65	11.2
***G. kola* extract**	6.34	8.6
***G. kola* powder**	5.85	8.2

The data revealed that the iso-α-acid contents of the *G. kola* ethanolic extract and *G. kola* powder were comparable to that of *H. lupulus* extract. The α-acid contents of the three hopping agents also showed that both *G. kola* extract and powder compared favourably with hop extract as shown in Table 1. These results suggest potential use of *G. kola* as a hopping agent in the brewing industries. The presence of α-acids and iso- α-acids is responsible for the bitterness of beer. The result is consistent with the observations of Okoro and Aina (2007).

Humulus lupulus and *Garcinia kola* have been known to exhibit some antimicrobial activity (Anichie and Uwakwe, 1990; Bishop, 1968). In this study extracts from these two plants were studied for their antimicrobial activity at various concentrations against a brewery wild strain of *S. cerevisiae*. *Garcinia kola* powder, *Garcinia kola* ethanolic extract and hop (*Humulus lupulus)* showed significant differences in their effects on the yeast's ability to grow (Fig. 1).

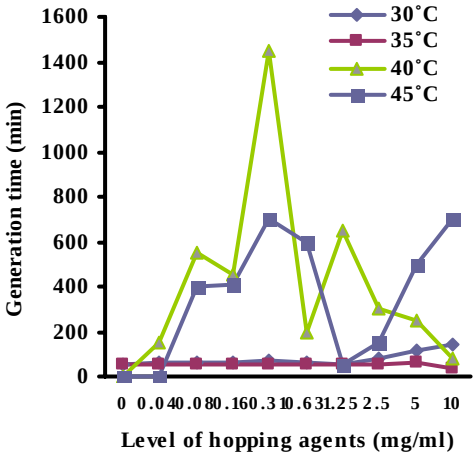

Fig. 1: Effect of *Garcinia kola* powder in the generation time of *Saccharomyces* wild yeast

Generally, the supplementation of the synthetic wort with *G. kola* (extract and powder) and *H. lupulus* hop caused an increase in yeast generation time of between 171.01 – 5270.07 % when compared to growth in the supplement – free medium. This suggests a possible inhibition of yeast growth by the hopping agents. The mean increase in yeast generation time was studied for the three hopping agents. Results showed that at 30 ^{0}C, yeast generation time increased by a mean value of 1286.85, 258.77 and 238.1 folds respectively when the wort was supplemented with *G. kola* ethanolic extracts, *H. lupulus* extract and *G. kola* powder (Fig. 2).

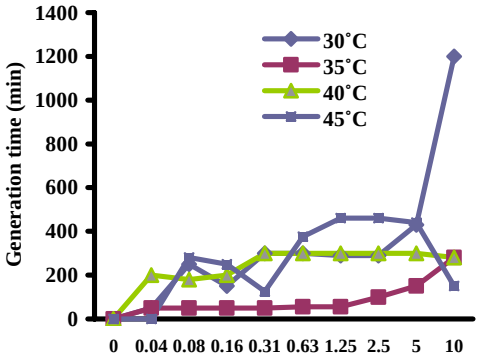

Fig. 2: Effect of *Garcinia kola* extract in the generation time of *Saccharomyces* wild yeast

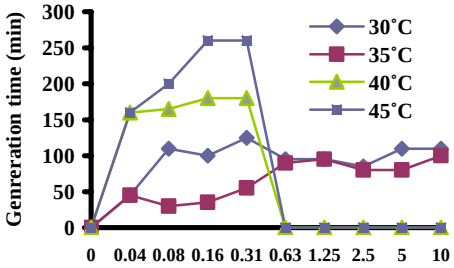

Fig. 3: Effect of hops (*Humulus lupulus*) extract in the generation time of *Saccharomyces* wild yeast

The results showed that *G. kola* ethanolic extract was the most effective antimicrobial agent at 30 ^{o}C, lowering the yeast growth rate by 498.4 and 540.47 times the value obtained for the *Humulus* extract and *G. kola* powder respectively (Fig 3). At 30 ^{o}C, the *H. lupulus* agent inhibited yeast growth 108.49% more than the *G. kola* powder (Fig. 4). Fig. 5 shows the relative effects of the hopping agents on yeast – generation time at 35 ^{0}C. The three agents also slowed down the rate of yeast multiplication but to a much lower extent (1.12 to 6.26 folds) than values (1.71 to 82.70 folds) obtained at 30 ^{o}C. This indicates that the agents were either less effective against the yeast at 35 ^{o}C or the yeast cells may have undergone some significant modifications at that temperature thereby making the cells less sensitive to the agents. Worthy of note is the observation that at 35 ^{o}C, lower concentrations of the hopping agents (0.039 mg/ml and 0.078 mg/ml respectively for *G. kola* extract and hop extract improved rather than inhibit the rate of growth of the yeast.

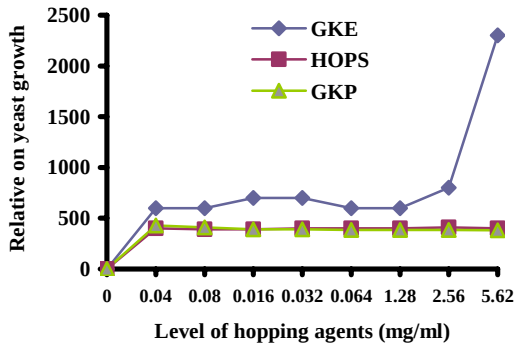

Fig. 4: Relative effect of hopping agents on yeast generation time at 30°C

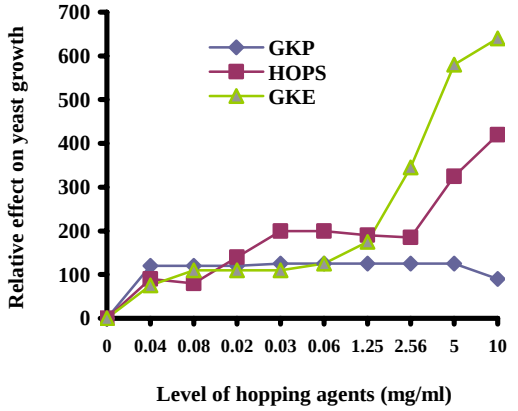

Fig. 5: Relative effect of hopping agent on yeast generation time at 35°C

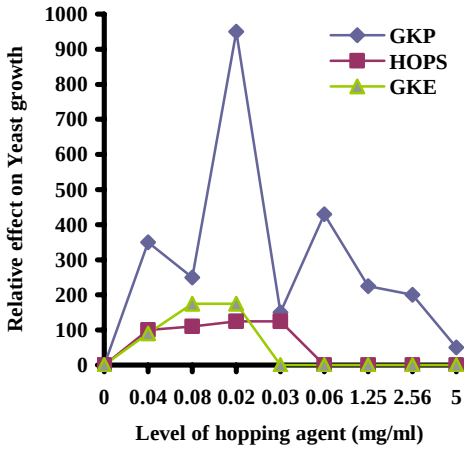

Fig. 6: Relative effect of hopping agents on yeast generation time at 40°C

While this behaviour cannot be readily explained, it is possible that the extracts contain some active components which are protective rather than inhibitory to the yeast at the higher temperature of 35 ^{0}C.

Fig. 5 indicates that at 30 ^{o}C, *K. kola* ethanolic extract had the highest mean inhibitory effect on yeast growth rate than hop and the *G. kola* powder. However, in contrast to the results obtained at 30 ^{0}C, where the *G. kola* extract gave highest values for inhibition at all the concentrations, it was observed that at 35 ^{0}C, *G. kola* powder was the most toxic of the three hopping agents at the two lowest concentrations evaluated. Hops was the most toxic among three middle concentrations (0.313 mg/ml, 0.625 mg/ml, 1.25 mg/ml) while *G. kola* extract gave the higher toxicity values than the other two agents at the three highest concentrations. While it is difficult to explain these results, it is possible that in each of the agents several compounds may be present whose interactions may play very influential role in the outcomes of the assay at the specific temperatures. Results presented in Fig. 5 also indicate that *G. kola* extract gave 1.325 – 2.045 times more activity than hops and *G. kola* powder respectively at 35^{0}C. Similarly, relative activity by hop was 1.54 folds greater than that of *G. kola* powder.

When the yeast cells were incubated at 40^{o}C in the presence of the hopping agents, total inhibition of the yeast growth by *G. kola* extract and hop extract was observed at concentrations above 0.039 mg/ml and 0.078 mg/ml, respectively. The fact that *G. kola* extract produced complete inhibition of growth at a much lower concentration than hops extract would seem to suggest that the extract of *G. kola* had more antimicrobial activity than either the hop extract or the *G. kola* powder employed in this study. The *G. kola* extract produced no complete inhibition at all the concentrations used at this temperature. It was however observed that at the very high concentrations it encouraged yeast growth, at rates well in excess of the value of the un-supplemented control (Fig. 6). Results at 45^{0}C behaved similarly except that at this temperature, the wild yeast was never completely inhibited by the *G. kola* extract. Conversely, the inhibition of growth by the hop extract was more pronounced with growth observed only at the last two concentrations of the agent (Fig. 7). These observations suggest that the effect of the *G. kola* extract was very temperature sensitive, 40^{0}C being the most effective. Akerele *et al.* (2008) reported good antimicrobial activity of ethanolic extract of *G. kola* against *Penicillium notatum*, *Aspergillus niger* and *Candida albicans*. Our observation is consistent with their result on the effectiveness of *G. kola* ethanolic extract against some spoilage organisms. The *G. kola* extract used in this study was a crude extract and it is possible that the interactions between components of the extract at 45^{0}C reduced the efficacy of the extract as an antimicrobial agent against the wild yeast of *Saccharomyces cerevisiae.*

Generally, the generation time of the wild yeast *Saccharomyces* for *G. kola* extract was in the order 40 ^{0}C > 30 ^{o}C > 45 ^{0}C. The relative effect of the hopping agents on the yeast generation time at 30 ^{0}C followed this order *G. kola* extract > Hop > *G. kola* powder with mean inhibition of 1286.85 %, 258.2 % and 238.1% respectively. At 35 ^{0}C the order of inhibition was *G. kola* extract > Hop > *G. kola* powder as obtained in 30^{0}C, though the mean inhibition was lower giving 254.71%, 192.24% and 124.58% respectively. Moreso, at 40 ^{0}C and 45^{0}C the order of inhibition were as follows: *G. kola*

extract > Hops > *G. kola* powder at concentration of

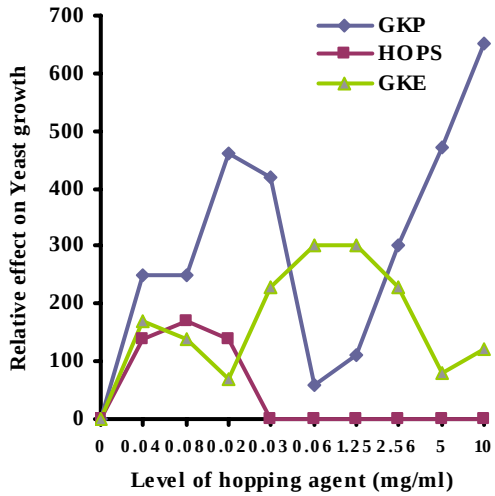

Fig. 7: Relative effect of hopping agents on Yeast generation time at 45°C

6.13 mg/L, 6.93 and 5.71 mg/L, respectively.

Conclusion: Results from this study show that the α-acid and iso-α-acid of both *G. kola* ethanolic extract and powder are highly comparable with hops. It is observed that the antimicrobial profile of *G. kola* ethanolic extract is as effective as that of hops against the brewery wild yeast, *Saccharomyces cerevisiae* (potential spoilage organism in the brewing industry). *G. kola* ethanolic extract demonstrated superior antimicrobial activity against the wild yeast than *G. kola* powder. *G. kola* ethanolic extract was more effective at 40°C. These data therefore indicate that *G. kola* either in the form of ethanolic extract or powder could serve as a good substitute for hops. Since *Garcinia kola* is readily available in Nigeria, a lot will be saved on the huge amount spent on the importation of hops if *Garcinia kola* is used in place of hops. The antimicrobial activity exhibited by *G. kola* extract and powder against *Saccharomyces cereviasie* makes *G. kola* a good substitute for hops in the brewing industry. The results of this study also revealed that the antimicrobial activity of *G. kola* extract against brewery wild yeast may be limited by its temperature dependence. Further work is suggested to unravel the cause of the decrease in the antimicrobial activity of *G. kola* at higher temperatures and possibly suggest ways to eliminate it.

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