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EVALUATION OF ANTI-NUTRITIONAL FACTORS IN HOP EXTRACTS AND EXTRACT FROM AZADIRACHTA INDICA AS POTENTIAL SUBSTITUTE IN THE NIGERIAN BEER INDUSTRY

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Abstract:-
The anti-nutritional factors in Azadirachta indica (neem) as contrasted to hops were carried out in terms of their methanolic extracts. These factors (trypsin inhibitors, phytate, cardiac glycoside, oxalate, cyanogenic glycoside and hydrogen cyanide) were screened using standard methods. The result of the assay revealed that trypsin inhibitors ranged from 6.45-17.30%, phytate ranged from 0.99% to 1.68% while cardiac glycoside ranged from 3.5% to 6.0% and the concentration of oxalate ranged from 0.0405mg/100g to 0.1020mg/100g, cyanogenic glycoside ranged from 0.216ppm to 0.810ppm while hydrogen cyanide ranged from 0.540ppm to 1.404ppm. It was established from ranking that A. indica was closer to isomerized hop extract than to hop leaf extract in the content of anti-nutritional factors. Hence, the extract from A. indica could be used as suitable substitute for hops in beer brewing.

Key words:-*Antinutritional, hops, A. indica, extracts, substitute*

INTRODUCTION

The anti-nutritional factors can be defined as those substances generated in natural food substances by the normal metabolism of species and by different mechanisms (e.g. inactivation of some nutrients, diminution of the digestive process or metabolic utilization of feed) which exert effects contrary to optimum nutrition. Anti-nutrients are found at some level in almost all foods as secondary metabolites for a variety of reasons (Philips, 993). Anti-nutritional factors such as trypsin inhibitor, phytic acid, oxalic acid and cyanogen are as important as nutritional content of any edible plant part and could improve people's health (Welch and Graham, 2004; Chavan and Kadam, 1989). Many traditional methods of food preparation such as malting, cooking and fermentation increase the nutritive quality of plant foods by reducing certain antinutrients (Holtz and Gibson, 2007; Oboh and Olodunmoye, 2007).

Beer brewing involves these processes (malting, wort boiling and fermentation). *Humulus lupulus* (hop) plants are vital to the brewing industry and some of their unique chemicals have the potential to be used in the nutraceutical industry (Shellie *et al*., 2009). Hop extracts give beer its bitter taste, improve foam stability, enhance aroma and flavour, and act as antiseptic towards microorganisms (Ashurst, 1971). Hops are grown throughout the temperate regions of the world to meet the demands of the brewing industries (Hough *et al*., 1982). Nigeria is in tropical region but beer production in Nigeria has increased recently due to ready markets and the importation of hops to meet the demand of the brewing industries becomes inevitable and continues to constitute a significant proportion of the Nigerian economy. Consequently, huge amounts of foreign exchange are being spent by this sector on importation of hops. The current economic recession demands that researches are geared towards the discovery and development of local raw materials to avoid huge waste of hard earned foreign exchange.

A lot of efforts have been made in the brewing industry for the substitution of barely with some local cereals. The substitution of hops with local raw materials has not received commensurate attention. This piece of work was designed to identify some ant nutritional factors in imported hops as well as contrast to *Azadirichta indica*. It was also designed to find the possibility of this plant serving as substitute for hops in beer production. This level of raw material freedom confers definite economic advantages to the Nigerian brewing industry.

Azadirachta (neem) is a genus of two species of trees in the Mahogany family, *Meliaceae*. Numerous species have been proposed for the genus but only two are currently recognized, *Azadirachta excelsa* and the more economically important tree, *Azadirachta indica* which is the only species in Nigeria (Keay*etal*., 1964). Products made from neem are found to be antifungal, antidiabetic, antibacterial, antiviral, contraceptive and sedative (Buttler and Bailey, 1973; Mabberley, 1995).One thing it has in common with hop is that it is bitter like hop but thrives in tropical regions, unlike hop (Ajebesone and Aina, 2004).

MATERIALS AND METHODS

Procurement of Materials

Hop leaf and isomerised hop extract were respectively purchased from Youngs **Ubrew** Goldings Hops and Ritchies both in theUnited Kingdom. The leaves of *A.indica* were obtained from the herbarium of Nnamdi Azikiwe University, Awka. Chemicals used were as detailed by AOAC, ASBC, and IOB.

Sample Preparation

Except for the isomerised hop extract prepared by Ritchies, each plant sample was milled and vacuum dried at 50^{o}C. Two kilograms (2kg) of each plant material thus prepared was stored in a dessicator for the rest of the experiment. Three hundred grams (300g) each of the resulting powders were then used to obtain methanolic extracts by steeping procedure.

Oxalates

This was determined according to Harbone (1995) as adopted by Osagie *et al*. (1996). This determination involves three major steps – digestion, oxalate precipitation and permanganate titration.

Digestion: 2g of the extract was suspended in 190cm^3 of distilled water in a 250cm^3 volumetric flask. 10cm^3 of 6M HCl was added and the suspension digested at 100^{o}C for 1 hour. The digested solution was allowed to cool, filtered and the filtrate received in another 250cm^3 volumetric flask. The residue was washed twice with 10cm^3 of deionized water and made up to the mark.

Oxalate Precipitation: Aliquot portions (125cm^3) of the filtrate were measured into two different beakers and four drops of methyl red indicator added to each beaker. This was followed by the addition of ammonia solution (dropwise) until the test solution changes from salmon pink colour to a faint yellow colour (pH 4 – 4.5). Each portion was then heated to 90^{o}C, cooled and filtered to remove precipitate containing ferrous ion. The filtrate was heated to 90^{o}C again and 10cm^3 of 5% $CaCl_2$ solution was added with constant stirring. After heating, it was cooled and left overnight at room temperature. The solution was then centrifuged at 2500 rpm for 5 minutes.

The supernatant was decanted and the precipitate completely dissolved in 10cm^3 of 20% (v/v) H_2SO_4 solution.

Permanganate Titration: The total filtrate resulting from digestion of 2g of the sample extract was made up to 300cm^3. Aliquots of 125cm^3 of the filtrate was heated until near boiling and then titrated with 0.05M $KMnO_4$ solution to a faint pink colour which persists for some 30 seconds.

The Calcium oxalate content is calculated using the formula:

$$\frac{T \times (VMe)(DF) \times 10^5}{(ME) \times (Mf)} \quad (ppm)$$

where T is the titre value of $KMnO_4$ (cm^3), VMe is the Volume – Mass equivalent (i.e. $1cm^3$ of 0.05M $KMnO_4$) solution is equivalent to 0.00225 anhydrous oxalic acid), DF is the dilution factor, $Vt/A = 2.4$ where Vt is the total volume of titrant ($300cm^3$) and A is the aliquot used ($125cm^3$), ME is the molar equivalent of $KMnO_4$ in oxalate and Mf is the mass of sample used.

Phytic acid

Phytic acid contents were determined using the method of Trease and Evans (1989) as adopted by Sofowora (1993). 2g of the different methanolic extracts was each weighed into different $250cm^3$ conical flasks. Each sample extract was soaked in $100cm^3$ of 2% concentrated HCl for 3 hours. The solutions were then filtered, through a double layer of hardened filter paper. Fifty centimeter cube ($50cm^3$) of each filtrate was placed in $250cm^3$ beaker and $100cm^3$ distilled water added to each of them. Ten cubic centimeter ($10cm^3$) of 0.3% ammonium thiocyanate solution was added as indicator and titrated with standard iron (III) chloride solution which contained 0.00195g iron per cm^3. The end point was slightly brownish – yellow which persisted for 5 minutes. The percentage Phytic acid was calculated using the formula;

$$\text{Phytic acid (\%)} = \frac{\text{Average titre value } \times 0.00195}{2} \times 100$$

Trypsin Inhibitor

Trypsin inhibitor activator of the samples was determined by the method of Harbone (1995). 2 g of each sample was weighed into a screw-cap centrifuge tube and $10cm^3$ of 0.1M phosphate buffer was added. The contents were shaken at room temperature for 1 hour on a UDY shaker.

The suspension obtained was centrifuged at 5000 rpm for 5 minutes and filtered through Whatman No. 42 filter paper. The volume of each was adjusted to 2ml with phosphate buffer. The test tubes were placed in water bath, maintained at 37°C and $6cm^3$ of 5% tricarboxylic acid (TCA) solution was added to one of the tubes to serve as a blank. Two ml casein solution was added to all the tubes previously kept at 37°C and were incubated for 20 minutes. The reaction was stopped after 20 minutes by adding $6cm^3$ of TCA solution to the experimental tubes and then shaken. The content was allowed to stand for 1 hour at room temperature. The mixture was filtered through Whatman No. 42 filter paper and the absorbance of filtrate from sample and trypsin standard solutions were read at 410nm.
Percentage trypsin inhibitor is expressed as follow: (Ologhobo and Fetuga, 1983).

$$\text{\% Trypsin inhibitors} = \frac{A^r_{410} - A^s_{410}}{A^r_{410}} \times 100$$

Where
A^r_{410} = Absorbance of reference at 410 nanometers
A^s_{410} = Absorbance of sample at 410 nanometers

Cardiac Glycoside

The method of Ayoola *et al*., (2006) was employed. To 2g each of the sample extract were added $50cm^3$ of distilled water and left for 48 hours, and then filtered with No. 42 Whatman filter paper. The filtrate was concentrated to a volume of $5cm^3$. To $1cm^3$ of the extract was added $1cm^3$ of 2% solution of 3,5-Dinitrosalicylic acid in methanol and $1cm^3$ of 5% aqueous NaOH. The mixture was boiled for 2 minutes (until brick-red precipitate was observed) and the boiled sample was filtered with a pre-weighed filter paper. The filter paper with the residue was dried in an oven to constant weight at 50°C. The weight of the filter paper with residue was taken. The cardiac glycoside was calculated in percentage as:

$$\%\ Cardiac\ glycoside = \frac{(Weight\ of\ filter\ paper + residue) - (Weight\ of\ filter\ paper)}{Weight\ of\ sample} \times 100$$

Cyanogenic Glycoside

10g of the sample extract was weighed into a $500cm^3$ round bottomed flask. To the sample extract was added $400cm^3$ of distilled water and heated on a bath for 2 hours. Distillation procedure was carried out and about $150cm^3$ of distillate was collected in a $250cm^3$ conical flask containing $20cm^3$ of 2.5% NaOH. To $100cm^3$ of the distillate containing cyanogenic glycoside $10cm^3$ of 6M NH_4OH and $2cm^3$ of 5% KI were added. The mixture was titrated with 0.02M Silver nitrate to a permanent turbidity to indicate the end point. Cyanogenic glycoside is obtained from the relation:
$1cm^3$ of 0.02M $AgNO_3$ = 0.54 Cyanogenic glycoside (ppm) (Bradbury *et al*., 1999).

Hydrogen Cyanide
The hydrocyanic acids (HCN) of the samples were determined using the procedure of Bradbury *et al* (1999). This was done by soaking 2g of each sample in 100cm^3 of water followed by the addition of 10cm^3 of concentrated orthophosphoric acid and left for 16 hours at a temperature of 38°C. Each sample extract was transferred into a two-necked 500cm^3 flask connected to a steam generator. This was steam-distilled with saturated sodium bicarbonate solution contained in a 50cm^3 conical flask for 60 minutes. To 20cm^3 of each distillate was added 1cm^3 of starch solution. The mixture was titrated with 0.2M iodine solution.Hydrogen Cyanide concentration was obtained from the relation: 1cm^3 of 0.2M iodine solution = 1.08 HCN (ppm)

Statistical Analysis
From triplicate experiments in anti-nutritional screening, the mean, standard deviation and were evaluated. The mean values were used to generate the tableof antinutritional results.
Simple statistics (ranking) was employed to determine the significant difference between the control (*A. indica*) and imported hops. In ranking, we determined the existence of the significant difference among *A.indica*, isomerized hop and hop leaf. We employed test of significant difference using one way Analysis of Variance (ANOVA). The software used for the analysis was SPSS (Special Package for Social Sciences) Version 20. In the test of significant difference, One Way Analysis of Variance (ANOVA) is the most suitable tool as it has the capacity to show the existence of difference at 5% level of significance (Gupta, 2011). In ANOVA, two hypotheses, H_0 and H_1 are stated and tested for:
H_0; there is no significant difference among samples of interest.
H_1; there is significant difference among samples of interest.

The result of the p- value (significance value) is used to accept or reject either of the hypotheses.

RESULTS AND DISCUSSIOIN
Oxalate, a conjugate base of the oxalic acid ($H_2C_2O_4$), is a naturally occurring substance found in plants and animals. It is a chelating agent for metal cations and thus has the ability to attract calcium cations to form calcium oxalate which causes nephrolithiasis (kidney stone). The toxicity of oxalic acid is due to kidney failure caused by precipitation of solid calcium oxalate (Curhan, 1999). Close examination of Table 1 shows that Oxalate concentration was comparatively close in all the samples. Therefore, *A. indica* can substitute hops Phytic acid was virtually of the same percentage range in all the samples but especially high in hop leaf and lowest in *A. indica*. Table 1 shows these relationships among the samples. Hence, *A. indica* and isomerized hop could substitute each other. Phytic acid, also known as inositol hexaphosphate (IP6), or phytate is a chelating agent that binds to minerals, metals or anything else it comes in contact with and takes them out of the body leading to loss of minerals in the body and its resultant consequences (Edman and Forbes, 1977). On the other hand, those authors reported that phytic acid is a powerful antioxidant as well as helpful in ridding the body of heavy metals and other toxins.

Trypsin inhibitors are chemicals that reduce the availability of biological active trypsin, an enzyme essential to nutrition of many animals, including humans. The trypsin inhibitors are reported to be one of the major toxic components of legumes (Liener and Kakade, 1980). The percentage trypsin inhibitor units was lowest in isomerized hop and highest in *A. indica* (Table 1). Based on these observations, *A. indica* can substitute imported hops in beer brewing when the concentration is somewhat decreased during hopping.

Table 1: Anti-nutritional Results of the Extracts

Phytochemicals	Isomerized Hop	Hop leaf	*A. indica*
Oxalate (mg/100g)	0.0405	0.0432	0.054
Phytate (%)	1.39	1.68	1.16
Trypsin inhibitor (%)	6.45	7.6	9.6
Cardiac glycoside (%)	4.5	3.5	6
Cyanogenic glycoside (ppm)	0.648	0.81	0.216
Hydrogen Cyanide (ppm)	0.756	0.648	0.54

Cardiac glycosides are organic compounds containing a glycoside that acts on the contractile force of the cardiac muscle and because of their potency in disrupting the functions of the heart, most are extremely toxic. These glycosides are found as secondary metabolites in several plants (Wang *et al*., 2008). From this study, it is evident that cardiac glycosides were especially high *A. indica* but comparatively similar in imported hops (Table 1). When the concentration of *A. indica* is decreased to a little extent during hopping, then this Nigerian bitter vegetable could substitute imported hops. Moreover,

therapeutic uses of cardiac glycosides primarily involve the treatment of cardiac failure, congestive heart failure, and as heart tonics, diuretics and emetics (Wang *et al*., 2008).

The concentration of cyanoglycoside was relatively low in *A. indica.* Table 1 shows that the concentrations of cyanogenic glycosides in imported hops did not differ much. Thus, all the samples could substitute one another. Cyanogenic glycosides or cyanoglycosides account for approximately 90% of the wider group plant toxins known as cyanogens (Agba-Egbe and Lape, 2006). Potential toxicity of cyanoglycosides arises from enzymatic degradation to produce free hydrogen cyanide (cyanogenesis), resulting in acute cyanide poisoning. Clinical symptoms of acute cyanide poisoning include rapid respiration; drop in blood pressure, rapid pulse, headache, dizziness, vomiting, diarrhoea, and mental confusion, blue discolouration of the skin due to lack of oxygen (cyanosis), twitching and convulsions (Davis, 1991; Haque 2002; Simeonova and Fishbein, 2004). The presence of cyanogenic glycoside in hops, though in very little amount may explain the reason why people who consume beers in excess sometimes vomit, complain of headache and feel dizzy.

The concentration of hydrogen cyanide in imported hops compares very well. It is evident from this work that the concentration of hydrogen cyanide in *A. indica* was lowests.
Hydrogen cyanide, sometimes called prussic acid is an inorganic compound. The chemical formula and chemical structure of Hydrogen cyanide are HCN and H – C □ N respectively. It is a colourless and extremely poisonous liquid that boils slightly above room temperature at 25.6ºC (http://www.wolfranalph.com/ %20input? =boiling point of +Hydrogen + cyanide). A hydrogen cyanide concentration of 3,500 ppm will kill a human in about 60 seconds (Vetter, 2000). The toxicity is caused by the cyanide ion, which halts cellular respiration by acting as a noncompetitive inhibitor for an enzyme in mitochondria called Cytochrome C oxidase (Patnaik, 2002; Gail *et al*., 2007).

Statistical Results
The p-value of the test is 0.878 (Table 4) which is higher than 0.05. We conclude that there is insignificant difference among the samples.

Table 2 ANOVA comparison of *A. indica* and imported hops

	Sum of squares	Df	Mean square	F	Sig.
Between Groups	458.306	4	103.459	0.288	0.921
Within Groups	17343.520	45	299.302		
Total	17801.825	49			

The output/result of the Post Hoc Test shows that hop leaf has the significance value of 0.877 followed byisomerized hop with significance value of 0.806 which are less than 0.921 but higher than 0.05. This shows that imported hops are not significantly different from *A.indica*. Hence, the closest substitute to *A.indica* is hop leaf. This implies that *A.indica* is closer to hop leaf extract as substitute than to isomerized hop extract.

CONCLUSIONS
This study has shown that the extracts from *A*.indica could be used as suitable substitute for hops in beer brewing. Extract of *A*.indica is closer as a substitute to isomerized hop extract than to hop leaf extract. Consequently, academic activities in the area of mixtures of hop extracts and extracts of *A.indica* which mimic very closely hop taste is strongly recommended.

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