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Genetic Diversity and Phytochemical Studies on Selected Ecotypes of Utasi (*Gongronema latifolium*) Plant Using Microsatellite Markers

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Diversity and phytochemical studies were carried out on twelve ecotypes of utasi, *Gongronema latifolium* using simple sequence repeats markers. Screening of ten (10) trinucleotide SSR primers, produced 4 primers that were able to amplify the DNA from all the plant ecotype. A total of 34 bands were amplified from the 4 SSR primers which include SSR 2, SSR 3, SSR 1 and SSR 4. Out of the amplified products using the 4 primers, 17 were found to be polymorphic with an average of 9 bands per primer. The number of amplification products per primer ranged between 10 in SSR 4 to 12 in SSR 2 with SSR 1 having the least number of bands (1). The sequences of the 4 random primers used in this study along with the number of bands generated and the number of polymorphic bands. The results of genetic analysis of the *Gongronema latifolium* ecotypes delineated the 12 ecotypes into five cluster groups. Results of phytochemical analysis of leaves of *Gongronema latifolium* showed (Table 2) mean saponin (2.09 ±0.01%), mean tannin (1.17 ± 0.01%), mean flavonoid (2.53 ±0.01%), mean polyphenol (4.60 ± 0.01%) and mean reducing

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compound (7.92 ± 0.01%).The phytochemical indices of the least 1.17± 0.01% and highest 7.92± 0.01% were constituted by tannins and reducing compounds, respectively. Thus, the study suggested the need for future exploitation of *Gongronema latifolium* leaves for phytochemical contents due to their high medicinal value.

Keywords: Genetic diversity; Gongronema latifolium; phytochemical analysis; microsatellite markers; DNA extraction.

1. INTRODUCTION

Gongronema.latifolium commonly known as Utazi and Arokeke by the South-South and South-West inhabitants in Nigeria is found in Africa, Asia and Oceania. It is a tropical phyto-protein that is used as spice. Ugochukwu and Babady [1]. Apart from the proteinous aspect of this plant, it is also used as a traditional medicinal plant due to its phytochemical composition for the treatment of various gastrointestinal disorders such as diarrhea, ulcers, dyspepsia and also in the management of diabetes mellitus [2,3].

"Following domestication, genetic diversity in plants has continued to narrow down due to a continuous selection pressure for specific traits i.e. yield, thus rendering them more vulnerable to disease and insect epidemics and jeopardizing the potential for sustained genetic improvement over a long term. Thus it is extremely important to study the genetic composition of the germplasm of existing modern-day cultivars in comparison with their related species. This will not only provide information on their phylogenetic relationship but will also indicate a chance of finding new and useful genes, as the ecotypes with the most distinct DNA profiles are likely to contain a great number of novel alleles" [4].

Genetic diversity, the level of biodiversity, refers to the total number of genetic characteristics in the genetic makeup of a species. It could also refer to both the vast number of different species as well as the diversity within a specie. Genetic diversity serves as a way for populations to adapt to changing environments. With more variation, it is more likely that some individuals in a population will possess variations of alleles that are suited for the environment. Those individuals are more likely to survive to produce offspring bearing that allele. The population will continue for more generations because of the success of these individuals.

"A 2007 study conducted by the National Science Foundation that genetic diversity and biodiversity are dependent upon each other, that diversity within a species is necessary to maintain diversity among species and vise versa. According to the lead researcher in the study, "if any one type is removed from the system, the cycle can break down, and the community becomes dominated by single species" [5].

Survival and adaptability of species bring about a high genetic diversity because when a species environment changes, slight gene variations are necessary to produce changes in the species anatomy that enables it to adapt and survive. Species that has a large degree of genetic diversity among its population will have more variations from which to choose the best fit alleles. Species that have very little genetic diversity are at a great risk because with very little genetic diversity within species, healthy reproduction becomes increasingly difficult, and offspring often deals with similar problems. The vulnerability of a population to certain types of diseases can also increase with reduction in genetic diversity.

Amongst the highly useful and medicinal important plants found in the Sub-Saharan regions of Africa, *Gongronema latifolium,* formerly called *Marsdenenia latifolium* Benth, is one of the most widely used species of its genus. It belongs to the Asclepiadaceae family. It is called "Utazi", "Utezi" and "Arokeke" in south eastern and western Nigeria, respectively. Ugochukwu and Babady [1].

"The plant is used as a leafy vegetable in south-eastern Nigeria and a good source of vitamins, protein, iron and minerals" [6]. "The medicinal importance of *G. latifolium* cannot be over emphasized. The plant plays a vital role in the treatment and prevention of varied health related problems including liver diseases, diabetes mellitus, high blood pressure, loss of appetite, dysentery, stomach pains, worm infectors, cough and malaria fever" (Agbo et al. 2005); [6]. "Medicinal importance of the plant is further elaborated by the presence of five bioactive compounds including alkaloids, saponnins,

tannins, flavonoids, and glycosides in leaves, which was suggested to proffer varied pharmacological effects on its specie" [7].

"Plants' improvement, either by natural selection or through the efforts of breeders, has always relied upon creating, evaluating and selecting the right combination of alleles. The manipulation of a large number of genes is often required for improvement of even the simplest of characteristics. With the use of molecular markers, it is now a routine to trace valuable alleles in segregating populations and mapping them. These markers once mapped, enable dissection of the complex trait into component genetic units more precisely, thus providing breeders with new tools to manage these complex units more efficiently in a breeding programme" [8].

"Molecular markers have been looked upon as tools for a large number of applications ranging from localization of a gene to improvement of plant varieties by marker-assisted selection. They have also become extremely popular markers for phylogenetic analysis adding new dimensions to the evolutionary theories. If we look at the history of the development of these markers, it is evident that they have been improved over the last two decades to provide easy, fast and automated assistance to scientists and breeders. Plants breeding analysis based on molecular markers has generated to preserve and popularize it" [9].

Microsatellite or simple sequence repeats markers are amplification products of anonymous DNA sequences using single, short di or tri oligonucleotide primers and thus do not require prior knowledge of a DNA sequence [10]. Low expense, efficiency in developing a large number of bands in a short time and requirement for less sophisticated equipment has made the microsatellite technique valuable especially for the studies of genetic diversity amongst crops.

Hence this study seeks to unveil the diversity in different species of Utazi (*Gongronema latifolium*) with regard to its medicinal and molecular characteristics using reliable genetic markers.

2. MATERIALS AND METHODS

2.1 Sample Collection

Young leaves of *Gongronema latifolum* obtained from different locations in Cross River, AkwaIbom, Oyo and Edo States, Nigeria, (Table 1) was taken to the International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria. They were given codes for easy identification and then stored in a cold room for future laboratory analysis.

Table 1. Sources of plant samples used for the study

Designation	Ecotypes	Coordinates /elevation
G1	Oyo State, Ibadan	$N06^0$ 12. 234 $E006^0$ 21.235
G2	Oyo State, Ibadan	$N06^0$ 05.135 $E006^0$ 25. 328
G3	Oyo State, Ibadan	$N06^0$ 40. 234 $E006^0$ 25.235
G4	Oyo State, Ibadan	$N06^0$ 15.221 $E006^0$ 45. 328
G5	AkwaIbom, IbiakpanIkotEkpene L.G.A, Aks (1)	N 03^0 09. 678 $E007^0$ 49.163, Elev. 99m
G6	AkwaIbomOkobo, IbionoIbom L.G.A, Aks	$N03^0$ 05. 236 $E007^0$ 51.100 Elev 86m
G7	AkwaIbomIkotEkpene Ii, IbionoIbom L.G.A, Aks	$N03^0$ 11. 108 $E07^0$ 55. 133 Elev 124m
G8	Cross River, Biological Science Block (1)	$N04^0$ 56.981 $E008^0$ 21.190 Elev. 32m
G9	Cross River, Biological Science Block (2)	$N04^0$ 56. 981 $E008^0$ 21.190 Elev. 32m
G10	Cross River, Old Odukpani Road	$N04^0$ 56.981 $E008^0$ 21. 191 Elev. 34m
G11	Edo State, EguareIrrua	$N06^0$ 44. 234 $E006^0$ 45.235
G12	Edo State, Ikhideu-Egoro	$N06^0$ 45.235 $E006^0$ 05. 328 Alt 373m

Fig. 1. Showing leaf samples of *Gongronemalatifolium*

2.2 Total Genomic DNA Extraction from Utazi Leaves

Genomic DNA was extracted from fresh leaves of each sample using a modified method of Stotharde et al. [11]. Leaves samples were macerated and total genomic DNA was extracted from each sample using CTAB extraction buffer containing 2-merccaptoethanol, hexadecyltrimethyl-ammonium bromide (CTAB) (solid), tris (hydroxyl-mehyl) amino-methane, ethlenediamine-tetraacetic acid, disodium salt solution (EDTA), and sodium chloride. Each sample was soaked in TE (10mM trisHCL and 1mM EDTA) to get rid of the remaining ethanol. Tissue from each of the leaves was placed in a sterile 1.5ml Eppendodorf tube, 500 $\mu l$of CTAB solution was added and 10 μl of proteinase was added too. The genomic DNA was extracted from the CTAB buffer by adding an equal volume of chloroform and isoamyl alcohol to each tube, the organic and the aqueous layers were gently mixed for 5mins and spun at 13,000 rpm for 20 mins. The upper aqueous layer was removed into another sterile eppendorf tube and equal volume of 100% ethanol was added, mixed and incubated at -20°c overnight to enhance DNA precipitation. It was spun again for 13,000 rpm for 20mins.The pellet was washed with 70% ethanol and spun for another 20 mins supernatant was removed and the pellets dried at room temperature.

2.3 DNA Quantification and Quality Determination

The quantity and quality of genomic DNA extracted were examined by comparing the template DNA isolated from samples with a DNA ladder (gene ruler) of 50bps to 2kbp for SSR in a 1% agarose gel using 1 x TBE buffer and viewed in a gel box (G: Box, Syngene). The concentration and quality were further determined at optical density (OD) readings of 260nm and 280nm using a Nano-drop spectrophotometer (Thermo Scientific Nano-Drop 2000C). The concentrations were used to guide the normalization of DNA of each sample at a concentration of 20ng/μL. Additionally, the ratio of OD 260/280 was provided by the Nano-drop which gave an indication of purity of the samples. Pure DNA has OD260/OD280 value of 1.8 and a deviation from this signifies the presence of contaminants that inhibit PCR reaction.

2.4 Polymerase Chain Reaction Optimization

Polymerase chain reaction was carried out in an Eppendorf Master Thermocycler (Eppendorf, company**,** USA). Each reaction tube was made of 100ng of template DNA, 2.5mM of MgCL2, 100µM of dNTPs, 1X *Taq* buffer, 20picomolarof 10-mer Primer, and 1 unit of *Taq* DNA polymerase made to a final volume of 20µl.DNA amplification was performed using the following thermal profile or sequence: 94°C for 5 min (35 Cycle); 94°C for 1 min, 37°C for 1 min, 72 °C for 2 min (35 cycles); final extension was performed at 72°C for 10 min (35 cycle) and samples were cooled at 4°C.PCR optimization was carried out using six selected DNA samples. A pre-mix containing dNTPs (dATPs, dCTPs, dGTPs and dTTPs), $MgCl_2$, Tris-HCl (pH 9.0), KCl and TaqDNA was used. A master mix containing 2μl of sterile distilled water, 0.5μl of 10pmoles

forward primer, 0.5*µ*L of 10pocomoles reverse primer, 5*µ*l premix and 2*µ*l of template DNA was prepared.

The diluted DNA samples were subjected to polymerase chain reaction (PCR) amplification using simple sequence repeat (SSR) markers.

2.5 Gel Electrophoresis of PCR Products

Leaves samples per marker were separated on 1.0% agarose gel at 80V for 40 minutes. Agarose powder was dissolved in Tris-borate EDTA (1x TBE) buffer by slowly boiling in a microwave oven. The agarose was allowed to cool and 1mg/ml concentration of ethidium bromide was added to the gel. The warm agarose solution was then poured into the gel tray in which combs were inserted to form sample wells. The gel was allowed to solidify for 30 minutes before immersing in the electrophoresis tank containing 100ml TBE buffer. The samples were run alongside 1.0*µ*L 1kb DNA ladder at 80 volts for 40 minutes. The amplified products were viewed under UV light in a gel box (G: Box, Syngene). Four of the primers showed amplification at various degrees while three primers did not amplify at all. Among the 12 primers that showed amplification, twelve (12) primers, six for each of SSR best were selected to amplify the polymorphicecotypes.

2.6 Phytochemical Analysis of the Plant Extracts of *G. latifolium*

Phytochemical analysis was carried out on the powdered and aqueous extract of samples of *G. latifolium* leaves using standard procedures to identify the constituents as described by Sofowara [12]. The phytochemical analysis was carried out to determine the presence of the following chemicals in the plant extracts: tannins, saponins, flavonoids and alkaloids.

2.6.1 Determination of Tannins

About 0.5g of the dried powdered sample was boiled in 20 .0 millimeters (ml) of water in test tube and then filtered. A few drops of 0.1 (%) ferric chloride were then added. Observation of a brownish green colour indicated the presence of tannins.

2.6.2 Determination of Saponins

About 2.0g of the powdered sample was boiled in 20.0ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with 5.0ml of distilled water and shaken vigorously for a stable persistent froth to occur. The frothing was mixed with 3 drops of olive oil and shaken vigorously. The formation of emulsion indicated the presence of saponin.

2.6.3 Determination of Flavonoids

1 ml. of 10.0% lead acetate solution was added to 1.0ml.of aqueous extract of the plant. The formation of a yellow precipitate indicated a positive test for flavonoids.

2.6.4 Determination of Alkaloids

3ml. of aqueous extract was stirred in 3ml. of 1% HCl on a steam bath. Mayer's and Wagner's reagents were added to the mixture. The turbidity of the resulting precipitate indicated the presence of alkaloids.

3. RESULTS

Genetic diversity is of great significance for breeding programmes as well as taxonomic studies. Molecular markers have frequently been used for the detection of genetic diversity in plants. It is mostly used because of its rapidity, simplicity and lack of any prior genetic information about the plant.

The SSR banding pattern of the 12 ecotypes of *Gongronema* are illustrated in Fig. 1. After screening 10 trinucleotide SSR primers, 4 primers were able to amplify the DNA from all the plant ecotype. A total of 34 bands were amplified from the 4 SSR primers which include SSR 2, SSR 3, SSR 1 and SSR 4. Out of the amplified products using the 4 primers, 17 were found to be polymorphic with an average of 9 bands per primer. Number of amplification products per primer ranged between 10 in SSR 4 to 12 in SSR 2 with SSR 1 having the least number of bands (1). The sequences of the 4 random primers used in this study along with the number of bands generated and the number of polymorphic bands are given in Table 2.

Ward linkage generated a dendrogram which illustrated the overall genetic diversities and relationships among the ecotypes surveyed. This is illustrated in Fig. 2.

The first cluster consisting of seven ecotypes of *G. latifolium* was further classified into three sub-clusters, 1A, 1B and 1C. "1A" consist of G9, G11

and G5, which were ecotypes gotten from Cross River, Edo State and Akwa Ibom State respectively.

"1B" consists of G6, G8 and G10. These were ecotypes gotten from Akwa Ibom (G6) and G8 and G10, collected from Cross River State. "1C" has an ecotype, G12 which was gotten from Edo State. (Fig. 2)

The second cluster contains two ecotypes G2 and G3 collected from Oyo State, Ibadan, while the third cluster has 3 ecotypes, G4, G7 and G1. These ecotypes G4 and G1 were gotten from Oyo State, Ibadan, while G7 was gotten from Akwa Ibom State.

It can be observed from the Dendrogram that even though the *G. latifolium* ecotypes, G9, G11 and G5 from Sub-cluster 1A were gotten from different locations, they had a genetic similarity. Morphologically the leaves and plant growth habits were the same; G11 had high altitudes of 373 as compared to G9 and G5 having low altitudes of 32m and 99m respectively. Also, the leaves of G9 and G5 were broad as compared to G11 having small leaves. This was because people from parts of Edo state were this ecotype was collected complained of lack of water, making most of the plants small and dark in colour.

Sub-cluster 1B, containing G6, G8 and G10 were ecotypes gotten from Akwa Ibom and Cross River and they formed a cluster, showing that they have a genetic similarity. Morphologically leaves were the same, broad, light and had thin stems.

The clustering pattern of G12, collected from Edo state indicates its genetic diversity, which might be due to its location. It had a very high Elevation of 405m above sea level as compared to other ecotypes.

Cluster 2 containing G2 and G3, collected from Oyo state, Ibadan clustered together showing its genetic relatedness amongst the two ecotypes. Also cluster 2 had G4, G7 and G1 collected from Oyo state and Akwa Ibom State. These three ecotypes clustered indicating their relatedness even if they were collected from different locations.

Ecotypes from Oyo State Ibadan from 001-04 were properly amplified ecotypes. This implies that SSR1 does not have a corresponding sequence with these ecotypes, hence resulting in a poor amplification product.

G5, G6, and G7 which were gotten from Akwa Ibom State produced poor amplification products using the primers especially in Ecotypes 5 and 6. Ecotype 7 was only amplified using SSR2 and SSR3, but SSR4, SSR1 produced no bands. This shows some level of genetic diversity as most of the primers do not have a corresponding sequence with the ecotypes.

Ecotypes 8, 9 and 10 collected from Cross River produced poor amplification products in SSR 3, SSR 4. SSR 2 produced bands in ecotype 8 and 10. SSR 1 produced bands in ecotype 10. This however shows genetic diversity amongst this ecotype as selected primers were able to amplify some ecotypes, produce unique bands or produce no band at all.

Ecotype 11 and 12 gotten from Edo State had an amplification product using SSR 1, SSR 3 and SSR 4. Only SSR 2 was able to produce reproducible bands in ecotype 12. SSR 2 therefore has a sequence that corresponds to ecotype 12 but does not correspond to ecotype 11. This shows some extent of diversity between the primers and the ecotype.

All the 4 primers produced a different number of polymorphic bands when used to screen the ecotypes. This explains the reasons for the diversity observed amongst *G. latifolium* ecotypes suggesting that the more polymorphic the bands are, the more diverse the plant. This also explains the variations in the chromosome. Aikpokpodion et al. [13].

3.1 Phytochemical Analysis of *Gongronema latifolium*

Phytochemical components of G.latifolium leaves were separated using High pressure liquid chromatography. Values were gotten using SPSS statiscal analysis.

Results of phytochemical analysis of leaves of *Gongronema latifolium* showed (Table 2) mean saponin (2.09 ±0.01%), mean tannin (1.17 ± 0.01%), mean flavonoid (2.53 ±0.01%), mean polyphenol (4.60 ± 0.01%) and mean reducing compound (7.92 ± 0.01%).The phytochemical indices of the least 1.17± 0.01% and highest 7.92± 0.01% were constituted by tannins and reducing compounds, respectively. The presence of these phytochemicals show the economic importance as well as the nutritional, therapeutic and industrial importance of *G. latifolium*.

Saponins, flavonoids, tannins all have high therapeutic advantages and as such, food or drug compounds supplemented with these chemicals have gained global significance due to the increased rate of life threatening diseases, cancer, diabetes, hypertension etc. Saponins have anticancer, antimalarial, antibacterial, analgesic and molluscidal properties [14]. They are responsible for other thereapeutic purposes including treatment of hypercalciuria in humans and as phyto protectants which preserve plants against microbial and fungal attacks. [15]. The presence of saponins however accounts for the bitter taste present in the leaves of G. latifolium [16].

Tsuchiya [17] reported a high range of biological activities in flavonoids from anti-inflammatory to antitumour characteristics for the treatment of neurodegenerative diseases. These phytochemical is present in G.latifolium and therefore suggests its therapeutic actions in certain diseases.

Physiological effects such as blood pressure reduction, serum lipid level reduction are expressed in tannins. They also show antibacterial, and anti-parasitic effects. Diseases such as diarrheae, rhinnorhoea have been treated with herbal medicine made from tannin-rich plants [18].

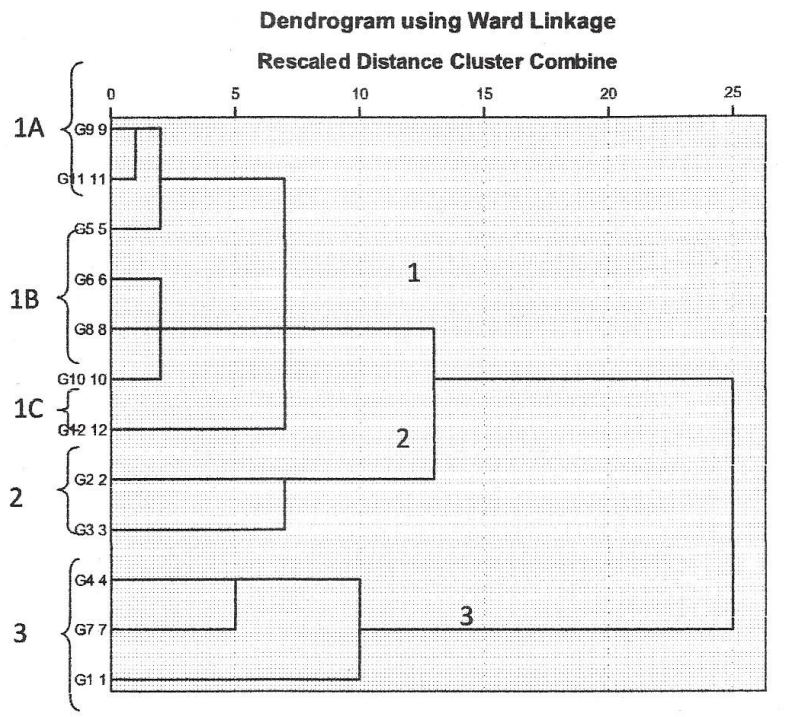

Fig. 2. Dendrogram showing genetic relationship between 12 Ecotypes of *Gongronema latifolium*

Table 2. Phytochemical profiles of *G.latifolium* leaf

Phytochemical Indices	Values (%)
Alkaloids	2.40 ± 0.06
Glycosides	2.26 ± 0.01
Saponins	2.09 ±0.01
Tannins	1.17 ± 0.01
Flavonoids	2.53 ±0.01
Polyphenol	4.60 ± 0.01
Reducing sugars	7.92 ± 0.01

4. DISCUSSION

Gongronerma latifoluim family Asclepediaceae is a creeping plant,geographically distributed in Africa, tropical and sub-tropical Asia and Oceania. In Nigeria, it is commonly called Utazi and arokeke in the south south and south west geopolitical zones. It is a tropical rain forest plant protein source used as vegetable spice Ugochukwu and Babady [1]. *Gongronema latifoluim* leaf meal flowers have revealed the presence of sure nutritional factors from the leaf, stem and root which varys in health applications respectively [19]. It has been used in the traditional system of medicine for various gastrointestinal disorders such as diarrhea, ulcers and dyspepsia and also in the management of diabetes mellitus [2], (Nwing, et al. 2005). The leaves have been reported to have a hypoglycaemic effect Ugochukwu and Babady [1] by decreasing activity of glucokinase enzyme and level of hepatic glycogen, and blood glucose. Phytochemcial studies of *G. latifolium* showed that the root contains polyphenol in abundance, alkaloids, glycosides and reducing sugar [20]. Mensah et al. [21] analysed the effect of *G.latifolum* on immune system of birds, and reported that it contained important compounds and served as antibiotics for treatment of common pathogenic strains of infective agents in birds. Further reports show its use for the prevention and treatment of diseases that could cause mortality in farm animals. Ugochukwu and Babady [1], aAgbo et al. [22].

Nutritionally, G. latifolium is rich in fats, protein, vitamins, minerals and essential amino acids [23]. Ani et al. [24] while feeding broiler birds onvarying dietary levels of *G. latifolium* leaf meal recorded a positive effect on growth performance of chicks at 75% level of *G.latifolium* leaf meal per 25kg of feed. Afolabi [25] investigated the chemical composition and anti-bacterial activity of *G. latifolium* and observed that *G. latifolium* was a good source of protein content at the rate of 27.2% in dry matter, high and compared favourably with percentage dry matter values reported for chickpea (24.0%), cowpea (24.7%), Lental (26.1%), fluted pumpkin leaves (22.4%) *Tamarndus indica* (24.3%),*Mucana flagellipies* (24.9).*Hibiscus esculantus* (23%) and *Parkiabig lobosa* (20.9%). Several previous and current studies on G. latifolium reported that it has nutritional high protein, vitamin and minerals. Akwaowo et al. [26]; Ajayi et al. [27] Iqbal et al, [28] Okafor [6] in their analyses of G. latifolium found that it was the cheapest most available source of important protein, vitamin, minerals and essential amino –acids that boost the physiological status of humans and promoted their growth. Moresibe et al [29] findings supported it as locally available and cheap plant protein source which is very important for future development of phytomedicine.

5. CONCLUSION

This study used molecular techniques to produce basic information on the genetically diverse nature of *Gongronema latifolium* ecotypes obtained from different ecologies. The results of genetic analysis further revealed that the utasi ecotypes shared common ancestral origin commonly segregated into five cluster groups. Phytochemical analysis of utasi ecotypes revealed non significant variations among the valuable presence of alkaloids, flavonoids, saponins, tannins, glycosides and reducing sugars which confer its medicinal properties. It has also shown that even though most plants are the same phenotypically and have same names, there is always a character (at the molecular level) that separates one plant from the other.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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