



TOXICOLOGICAL, PHYTOCHEMICAL, AND ANTIBACTERIAL ASSESSMENT OF *Chlorella vulgaris* AND *Spirulina platensis* POWDER IN ALBINO RATS. A PRELIMINARY STUDY

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ABSTRACT

Objectives. To describe acute toxicity, antibacterial activity and phytochemical assessment of *Chlorella vulgaris* and *Spirulina platensis* powders. **Material and Methods.** FeCl₃ test, Wagner test, Keller Killiani test, frothing test, alkaline solution and dilute acid; concentrated sulphuric acid were used for phytochemical analysis. Antibacterial screening of the extracts was conducted using the disc gel diffusion method in *E. coli*, *S. aureus* and *B. cereus* clinical strains. In order to evaluate acute toxicity and its effects on haematological and biochemical parameters; 15 albino rats were grouped into five groups: I (powder of aqueous extract of *Chlorella vulgaris*), II (powder of methanol extract of *Chlorella vulgaris*), III (powder of aqueous extract of *Spirulina platensis*), IV (powder of methanol extract of *Spirulina platensis*) and V (control). The dosage was 25g/day/rat. After six days, haematological and biochemical parameters and gross pathologic changes were evaluated. **Results.** Alkaloids and flavonoids were detected from the methanol extracts of both *Chlorella vulgaris* and *Spirulina platensis* (*Arthrospira*). Only cardiac glycosides and steroids were detected from *Spirulina*'s extracts. *Chlorella vulgaris* extracts significantly inhibited *B. cereus*. Rats fed with *Chlorella vulgaris* and *Spirulina platensis* powder showed an increase in white blood cell counts and haemoglobin level compared to negative control rats ($p < 0.001$). Serum glutamic oxalate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) had normal values but significant differences between groups ($p < 0.001$). **Conclusion.** This powder is rich in bioactive phytochemicals but only *Chlorella*'s extracts have shown antibacterial effect. Signs of toxicity weren't found in any parameter.

Keywords: *Chlorella vulgaris*; Phytochemicals; Disk Diffusion Antimicrobial Tests; Plant Extracts/toxicity (Source: MeSH)

RESUMEN

Objetivos. Describir la toxicidad aguda, efecto antibacteriano y análisis fitoquímico de los polvos de *Chlorella vulgaris* y *Spirulina platensis*. **Materiales y métodos.** Se realizaron las pruebas de FeCl₃, Keller Killiani, de saponinas, solución alcalina y de concentración de ácido sulfúrico para el análisis fitoquímico. El efecto antibacteriano de los extractos fue evaluado mediante el método de difusión con discos en cepas de *E. coli*, *S. aureus* y *B. cereus*. Para evaluar la toxicidad aguda y los efectos del polvo en parámetros hematológicos y bioquímicos, se agruparon 15 ratas albinas en cinco grupos: I (polvo de extracto acuoso de *Chlorella vulgaris*); II (polvo de extracto metanólico de *Chlorella vulgaris*); III (polvo de extracto acuoso de *Spirulina platensis*); IV (polvo de extracto metanólico de *Spirulina platensis*), y V (grupo control). La dosis usada fue de 25 g/día/rata. Después de seis días, se evaluaron todos los parámetros y cambios macroscópicos en los órganos. **Resultados.** Se encontraron alcaloides y flavonoides en los extractos metanólicos de *Chlorella vulgaris* y *Spirulina platensis* (*Arthrospira*). Se detectaron glucósidos cardíacos y esteroides en los extractos de *Spirulina platensis*. Los extractos de *Chlorella vulgaris* inhibieron el crecimiento de *Bacillus cereus*. Las ratas alimentadas con los polvos de *Chlorella vulgaris* y *Spirulina platensis* incrementaron el conteo de leucocitos y los valores de hemoglobina comparados con el grupo control ($p < 0.001$). Las transaminasas (SGOT y SGPT) se encontraron en valores normales, pero con diferencias significativas entre los grupos ($p < 0.001$). **Conclusiones.** Estos polvos son ricos en componentes fitoquímicos activos, pero solo los extractos de *Chlorella vulgaris* mostraron efecto antibacteriano. No se encontraron signos de toxicidad aguda en ningún parámetro.

Palabras clave: *Chlorella vulgaris*; Fitoquímicos; Pruebas Antimicrobianas de Difusión por Disco; Extractos Vegetales/toxicidad (Fuente: DeCS Bireme)

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BACKGROUND

Lower plants such as algae consist of a tremendous variety of secondary metabolites that has been exploited as potential drugs. The interest in studying the algae has increased since Robertson and Fong (1940) reported the antibacterial properties sign of *Chlorella vulgaris*. Many bioactive compounds in algae have been found to have the anti-coagulant properties, anti-platelet and anti-tuberculosis potential ⁽¹⁾. Despite the uses of algae extracts for the pharmaceutical applications, researchers have focussed their interest solely in the algae extracts for antimicrobial activity, cultivation studies for biofuels, with little studies on toxicology and the effect of microalgae on haematological parameters. *Chlorella vulgaris* and *Arthrospira* (common name: *Spirulina*) is widely used as a human health supplement and also as animal feed due to its high protein content and high concentration of essential amino acids, vitamins, minerals and fatty acids ⁽²⁾. Additionally, *Chlorella vulgaris* and *Arthrospira* has been shown to possess a range of therapeutic properties. The detection of primary and secondary metabolites in these two algae has been employed in various pharmaceutical applications. Secondary metabolites of these algae have the potential as an antiviral, antineoplastic, antibacterial, anti-HIV, anti-inflammatory, anti-tumour as well as anti-anaphylactic agents ⁽³⁻⁷⁾.

A recent study of the methanol and water extracts of *Chlorella Vulgaris* and *spirulina platensis* was evaluated. The detail phytochemical constituents, toxicology and effects of oral consumption of *Chlorella vulgaris* and *Spirulina platensis* have not been evaluated. Kumar et al. ⁽⁸⁾ also stated that the importance of analysing the phytochemical constituents is due to the fact that these chemicals are the key on the biological activities. Although many studies showed the extensive use of these microalgae, its effects on haematological parameters and cytotoxicity were unknown.

Therefore, in this study, the antibacterial activity, preliminary phytochemical, toxicological screening and haematological/biochemical effects of *Chlorella vulgaris* and *Spirulina platensis* (*Arthrospira*) were investigated.

MATERIAL AND METHODS

Chlorella vulgaris and *Spirulina platensis* were obtained from Chinese health shops (Tianshi shop) in Bamenda (Cameroon) produced and marketed by Green SuperFoods. Samples were taxonomically identified by botanists at the Institute for Agronomic Research Bambui, in Cameroon, with a voucher sample deposited as PRF01SP at the Phytobiotechnology Research

Laboratories. A sample each of 40 g of the pelletised algae was added to 200 ml of methanol and water separately. The set ups were allowed to sit for a period of 48 hours and each set up was filtered by gravity. The solvents were evaporated in a rotary evaporator.

Phytochemical analysis

All of the extracts were subjected to preliminary phytochemical screening which described by Trease & Evans (1989) and Harborne (1998) ^(9,10). The preliminary screenings were carried out to identify alkaloids, tannins, saponins, phlobatannins, cardiac glycosides, steroids, triterpenoids and flavonoids and phlobbatannins, the Liebermann-Burchard's test was used. For tannins, FeCl₃ test was used; for alkaloids, Wagner test was used; for cardiac glycosides, Keller killiani test was used; for saponins, frothing test was used; for flavonoids, alkaline solution and dilute acid were used for testing; for steroids and terpenoids, chloroform and concentrated sulphuric acid was used for testing.

Antibacterial testing

Antibacterial screening of the extracts was conducted using the disc gel diffusion method ⁽¹¹⁾. Tested organism (*Escherichia coli*) was selected and grown in nutrient agar at 37°C for 24 hours prior to the testing. The extracts were dissolved in its extracting solvent and made up to a concentration of 40 mg/ml. Each of the bacterial isolates was then subcultured in another nutrient agar and spread it evenly using the sterile bent metal over the surface of an agar plate. After inoculation, the discs (6 mm diameter) were made and immersed with 0.2 ml of extracts. The extracting solvents, such as methanol and water; were included as a positive control. The Petri-plates were then incubated at 37 °C for 24 hours. The actual growth inhibition of the test organism was examined by measuring the diameter of inhibition zones for each extract and compared it with the diameter of inhibition zones for each control.

Toxicological and Haematological Methods

Laboratory animals were raised at the Phytobiotechnology Research laboratory, and fed with the test samples (*Chlorella vulgaris* and *Spirulina platensis*) separately for 6 days and bled for blood samples each. Samples were then analysed for haematological values manually and with automated haemoalyser. Gross anatomy was done to verify for signs of organ toxicity. The entire study was done in the Phytobiotechnology Research laboratory Bamenda, Cameroon, from June to August 2015.

A dose of 25g/day/rat of powdered *Chlorella vulgaris* and *Spirulina platensis* were considered to be the

minimum dosage to be given per day. A total of 15 rats (of approximately the same age: 3.5 to 4 months and weight (300 g) grouped into 5 groups: I (powder of aqueous extract of *Chlorella vulgaris*), II (powder of methanol extract of *Chlorella vulgaris*), III (powder of aqueous extract of *Spirulina platensis*), IV (powder of methanol extract of *Spirulina platensis*) and V (control), according to other studies (12). To control group, only the feed (25 mg/group/day) was administered for the duration of feeding. To the test groups (I, II, III and IV): 2 preparations of 65g of powdered plant and 130 g of normal feed was made from which 25 g was administered orally/day, moistened with clean water. Administration was done first thing in the morning.

After six days' administration, blood samples were obtained by exsanguinations and put into lithium heparin test tubes for haematological examination. All rats were examined carefully for gross pathologic changes, with particular emphasis on the liver, kidney, lungs, heart and spleen. All values were done using automatic blood analysers and results confirmed by manual methods. The mean values for each parameter was established. All tests were done in duplicate to minimize error.

Packed cell volume (PCV) Estimation

The packed cell volume, also called haematocrit, was used to calculate the Mean Cell Haemoglobin Concentration (MCHC) and Mean Cell Volume (MCV). These red cell indices are useful in the differentiation for the different types of anaemia. The packed cell volume is that proportion of whole blood occupied by the red cells, expressed as a ratio. Anticoagulated blood in a glass capillary of specified length bore size, and wall thickness is centrifuged in a microhaematocrit centrifuge at 12000 – 15000 rpm for 3-5 minutes to obtain constant packing of the red cells. A small amount of plasma remains trapped between the packed red cells. The PCV value is read from the scale of a microhaematocrit reader or calculated by dividing the height of the red cells column by the total column of blood.

Haemoglobin (Hb) estimation

Haemoglobin is the oxygen-carrying elements in the body. They are heterogeneous proteins produced in developing erythroblasts. Each human Haemoglobin (Hb) consists of a tetramer of 2 α and 2 β globin chains bound to a single heme moiety. Heme contains one ferrous iron (Fe²⁺) atom carried in a porphyrin ring.

From the relation $PCV = 3 \times Hb$, the Hb is directly calculated for each sample indicating that $Hb = PCV/3$.

$$Hb \text{ (g/dl)} = \frac{PCV \text{ (\%)}}{3}$$

White Blood Cell (WBC) Analysis

In this case, the WBC count is used to investigate possible leucopenia or leukocytosis due to the presence of the feed, and not merely the investigation of infections. Whole blood is diluted 1 in 20 in an acid reagent which haemolyses the red cells (not the nucleus of nucleated red cells), leaving the white cells to be counted. White cells were recounted microscopically using an improved Neubauer ruled counting chamber (haemocytometer) and the number of WBCs per litter of blood calculated.

A 0.35 ml of the diluting fluid into a term tube using the 1ml graduated pipette was prepared. Using the 20 μ l pipette; 0.02 ml of a sample of capillary blood or EDTA Venus blood was added. The blood was expelled 3 times in the diluting fluid by squeezing and releasing the rubber tubing. It was mixed by gently tapping the bottom of the tube a few times the dilution of the blood now was 1:20. The test tube was gently tapped to mix the diluted blood. The counting chamber was filled with the diluted blood using the Pasteur pipette. Air bubbles were avoided the counting chamber not over filled. The counting chamber was placed on the microscope stage to allow the cells to settle for 2 minutes. The counting chamber was well focused. The cells in 4 large squares of the counting chamber were counted.

Statistical Analysis

Comparisons among Hb, VCM, WBC, SGOT and SGPT values between groups were performed by one-way analysis of variance (ANOVA or Kruskal-Wallis) and Bonferroni's test as post-hoc test. Values of $P < 0.05$ were considered significant. Statistical analyses were performed using the statistical software STATA version 13 for Windows.

RESULTS

The nature of the methanol and aqueous extracts of *Spirulina platensis* and *Chlorella vulgaris* is, generally, greenish to dark green (Table 1). No antibacterial activity on the tested isolates was observed for *Spirulina platensis*.

Chlorella vulgaris extracts significantly inhibited only with methanol extract having the highest zones of inhibition ($X=21$ mm) as opposed to aqueous extract ($X=16$ mm). Extracts of *Spirulina platensis* and the extracting solvent controls did not exhibit any zone of inhibition.

Table 1. The Qualitative description of methanol and aqueous extracts of *Spirulina platensis* and *Chlorella vulgaris*.

<i>Spirulina platensis</i>			<i>Chlorella vulgaris</i>	
	Colour	Appearance	Colour	Appearance
Methanol	Dark greenish	Solid, sticky	Dark green	Solid, greasy
Aqueous	Dark brownish black	Viscous, smelly	Dark green	Viscous, smelly

Preliminary screening of extracts for alkaloids, tannins, saponins, cardiac glycosides, steroids, terpenoids and flavonoids was evaluated and results shown in Table 2. The phytochemical screening of the aqueous extracts of *Spirulina platensis* revealed that more of the phytochemical compounds were obtained, while for *Chlorella vulgaris*, the methanol extracted more phytochemicals.

Table 2. Secondary metabolites of methanol and aqueous extracts of *Spirulina platensis* (SP) and *Chlorella vulgaris* (CV)

Secondary metabolite	SP _M	SP _{Aq}	CV _M	CV _{Aq}
Alkaloids	-	-	+	+
Saponins	+	-	+	-
Cardiac glycosides	-	+	-	-
Steroids	-	+	-	-
Terpenoids	-	-	-	-
Flavonoids	+	-	+	+
Tannins	-	+	-	+

SP indicates *Spirulina platensis*, CV indicates *Chlorella vulgaris*. Subscripts M denotes Methanol extracts; Aq denotes Water extracts

Significant differences ($p < 0.001$) were found in comparison of haematological parameters amongst tests groups and control group; except in comparisons between groups I-II and III-IV (groups of different extracts from same plant). Serum glutamic oxalate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) had normal values in all groups, but they have significant difference between all groups ($p < 0.001$). (Table 3 and Figure 1). Differences in gross pathology between groups weren't founded.

DISCUSSION

The nature of the methanol and aqueous extracts of *Spirulina platensis* and *Chlorella vulgaris* is, generally, greenish to dark green. No antibacterial activity on the tested isolates was observed for *Spirulina platensis*. Both methanol and aqueous extracts of *Chlorella vulgaris* exhibited antibacterial activity on Gram-positive bacilli only. The potential antimicrobial active compounds are mostly found from the organic solvent extracts^(12,13). Although, no antibacterial activity was observed, previous studies also showed that the organic solvent extracts from *Spirulina platensis* have been found to show better antimicrobial activity⁽¹⁴⁾. In this study, all the extracts did not display any activity against *E. coli*. The finding in this study does not corroborate with the previous statement, is probably due to the influence of genetic and environment conditions and /or the strains of the test organisms used.

The preliminary phytochemical screening of the aqueous extracts of *Spirulina platensis* revealed that more of the phytochemical compounds were obtained; while for *Chlorella vulgaris* the methanol extracted more phytochemicals. This finding, possibly, lends credence to the reason why most researchers prefer to use water and methanol extracts of algae for the antimicrobial testing.

Bioactive compounds from algae showed to have the hypocholesterolemic, immunostimulatory, anti-inflammatory, anti-diabetic, anti-viral, anti-fungal and anti-bacterial properties that have been reported⁽¹⁵⁻²¹⁾.

From our results, cardiac glycosides were extracted by aqueous extraction from the *Spirulina platensis*. The presence of this compound premised that the aqueous extract of *Spirulina platensis* might have the potential

Table 3. Comparison of means of hematological and biochemical parameters between groups.

Parameters	I	II	III	IV	V	p value
Hb (g/dl)	11.80±0.10	11.88±0.03	11.20±0.10	11.20±0.01	10±0.00	0.001*
PCV (%)	35.72±0.04	35.76±0.05	33.55±0.05	33.59±0.12	30.01±0.01	0.001*
WBC (103/ul)	5.4±0.01	5.4±0.01	5.2±0.10	5.17±0.06	4.19±0.01	0.001*
SGOT	25.86±0.05	26.68±0.16	27.56±0.04	29.21±0.03	29.99±0.01	<0.001 ¶
SGPT	10.56±0.05	11.52±0.07	12.55±0.30	13.27±0.03	14.99±0.01	<0.001 ¶

*ANOVA was used, ¶ Kruskal-Wallis was used.

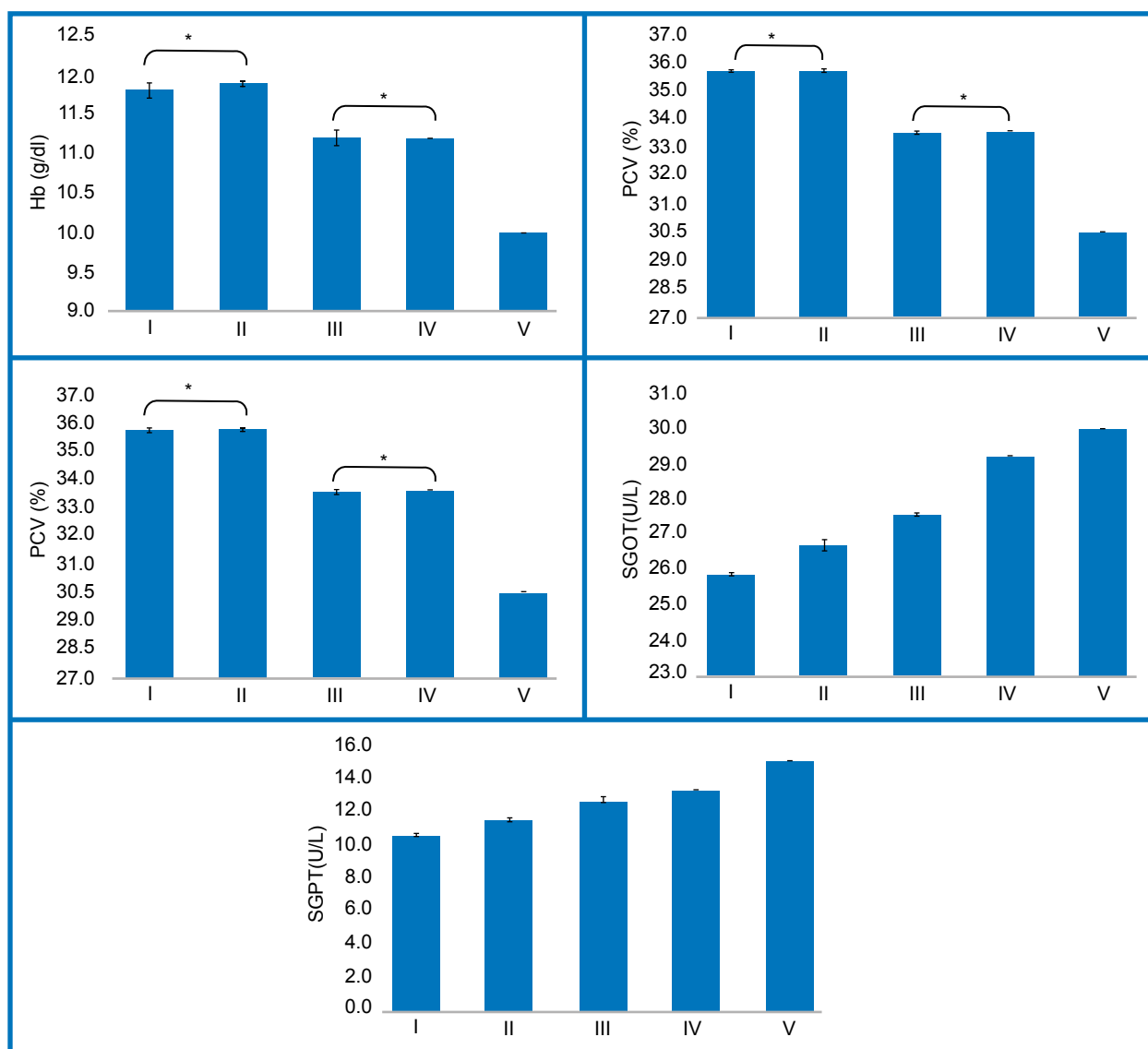


Figure 1. ANOVA plus Bonferroni analysis of comparisons of hematological and biochemical parameters. (*) Significant difference between means of groups was founded with Bonferroni analysis.

in lowering the blood pressure and showed to have significant effect in strengthening a failing heart ⁽²⁴⁾. Tannins have been found to have the anti-inflammatory and anti-bacterial effects ⁽²⁵⁻²⁷⁾.

Steroids found to have the similar role as saponins, which both of these bioactive compounds are responsible for central nervous system activities ⁽¹⁵⁾. In addition, steroids showed to possess analgesic and anti-inflammatory effect ^(19,28,29). A previous study reported that the sterols of *Spirulina maxima* are related to the antimicrobial activity ⁽³⁰⁾. This results did not support this finding, since the water extracts from *Spirulina* showed no antibacterial activity on the tested bacteria.

Numerous studies showed that the flavonoids have anti-tumour, anti-HIV and anti-inflammatory effect ^(31,32).

Flavonoids isolated from citrus fruits also demonstrated the anticancer both in vivo and in vitro activity ⁽³³⁾. From results, the presence of flavonoids was detected only in the methanol extract of *Spirulina platensis* while it was detected in both methanol and water extracts of *Chlorella vulgaris*. To further validate the therapeutic potential of these two algae, their effect on blood as well as their toxicity was evaluated using albino rats. In our study, after 6 days of administration gross anatomical examination did not show any sign of toxicity.

Significant elevation in the levels of Hb, WBC, and VCM in comparison to control group; agrees with previous results reported by other authors on the rich phytochemistry of these algae ^(22,23). Analytical reports of the algae suggest that they contain Vitamin A, beta-carotene, Thiamine (vit. B1), Riboflavin (Vit. B2), Niacin (vit. B3) Pantothenic acid (B5) Vitamin B6, Folate (vit. B9) Vitamin C, Vitamin

E, Calcium, Iron, Magnesium, Phosphorus, Potassium, Sodium, and Zinc significantly Iron, Vit. B6, B9 and other bioactive ingredients. These ingredients are vital for the formation of WBC and Hb ⁽¹³⁾.

Gross anatomical examination of organs in situ after dissection revealed no significant change on the organs from the control rats indicative of the fact those these algae was not toxic to the cells of the rats. The findings were further proved by the results of the liver enzymes profile which were generally lower than values from the control rats. But, we suggest a more adequate analysis and a complete toxicological study for a future studies.

In conclusion, this study suggests that these algae are non-toxic when consumed and no sign of toxicity (in haematological or biochemical parameters) was observed in the test rats. The studied extractions have a rich phytochemical composition: alkaloids, cardiac glycosides, saponins, as well as the antibacterial effect of *Chlorella vulgaris*, specifically on Gram-positive bacilli. This study is a preliminary analysis of some important characteristics of these products. So, we suggest a more complete study of these algae dietary supplements, because they have a frequent use and sales by foreign pharmaceuticals in sub Saharan Africa, particularly, in Cameroon.

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