

Full Length Research Paper

On the production of CONAVIR[®] immune-booster by good manufacturing practice: Development of specifications for the herbal component

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CONAVIR[®] is an immunostimulant phytomedicine developed at the National Institute for Pharmaceutical Research and Development, Abuja. The paper describes the steps taken and the results obtained in the attempt to develop specifications for the herbal component – the aerial parts of *Andrographis paniculata* Ness (Family: *Acanthaceae*), grown in the Institute's gardens. The said steps are vital for production as per good manufacturing practice. Qualitative and quantitative tests on the fresh and air-dried materials were carried out as prescribed in standard texts. Quantitative results on the dried herb included: bitterness value, $2.86 \pm 1.74 \times 10^3$ units per g; loss on drying, 10.64 ± 0.36 %w/w; total ash, 14.10 ± 4.49 %w/w; acid insoluble ash, 1.00 ± 0.06 %w/w; and water extractable matter, 30.37 ± 2.63 %w/w. Tests for phytochemicals revealed the presence of glycosides, saponins, tannins and alkaloids, but not of anthraquinones. Normal phase thin layer chromatography of the aqueous or ethanolic extract gave 5 spots, while the reverse phase of the same extracts gave 6. These results, including macroscopic and sensory examinations of the fresh herb, provided the quantitative and descriptive data needed for identifying and characterizing the herbal component of CONAVIR[®].

Key words: Conavir, immune-booster, phytomedicine, specifications, herbal component, *Andrographis paniculata*, *Acanthaceae*, good manufacturing practice (GMP).

INTRODUCTION

During the 1990's, when there was still a great dearth of affordable intervention in the control of HIV-AIDS in Nigeria, pressure was mounted on National Institute for Pharmaceutical Research and Development (NIPRD) NIPRD to meet the challenge. This was against the background of the success recorded with Niprisan, the sickle cell drug developed by the Institute. Among the many approaches considered by NIPRD was the development of CONAVIR[®] Conavir[®], an immunostimulant phytomedicine containing *Andrographis paniculata* (Inyang, 2009). A key step in the venture was

to domesticate the herb, which is of Asian origin. The Institute arranged for the seeds to be procured from India, and attempts to cultivate the plant in NIPRD's gardens began in the late 1990's. The result is a modest plantation of the Asian herb in Abuja, Nigeria, from which the material for this study was harvested. In order to systematize and scale up the production of CONAVIR[®], it is necessary to determine and ascribe quality specifications to its herbal component as required by good manufacturing practice (GMP) and by Nigeria's National Agency for Food and Drug Administration and Control (NAFDAC). Phytomedicine is one of the synonyms of "herbal product" or "herbal preparation", and is defined by NAFDAC as "regulated products of plant origin" (Akunyili, 2002). Thus, CONAVIR[®] is described as a phytomedicine rather than an herbal drug because of what it contains

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and how it is produced. This present study focuses on identification and characterization the fresh plant, on the one hand, and the air-dried material, on the other, for the purpose of qualifying the dried material for use in production. *Andrographis paniculata* is an exceedingly bitter herb that has been in use for thousands of years in Asia, where it is regarded as the "King of Bitters" (Coon and Ernst, 2004; Altancer.com, 2007). It grows erect to a height of over one meter in moist shady habitats, is widespread in most of Asia, and is popular in both Ayurvedic and Chinese Medicine (A-Zherbs, 2006).

The aqueous extract has activity against *Salmonella* and *Candida*, and is reported to be antihepatitic, antihepatotoxic, antibacterial, antifungal, antimalarial, anti-HIV, antithrombogenic, antiinflammatory and antipyretic (Altancer.com, 2007). The herb is also famed as an immunostimulant (Puri et al., 1993). The principal agent, andrographolide, first isolated by Gorter in 1911, is mostly extracted from the leaves (Altancer.com, 2007; Sharma et al., 1992). Andrographolide is an intensely bitter, water-soluble lactone that protects rats against carbon tetrachloride induced hepatotoxicity (A-Zherbs, 2006).

The LD50 in male mice is 11.46 gm/kg. Immunostimulatory activity of andrographolide is evidenced by increased proliferation of lymphocytes and interleukin 2 (Coon and Ernst, 2004; Altancer.com, 2007). The herb reproduces by seeds sown in March or April in Abuja, and begins to flower in August or September. The present paper focuses on the characterization of the Abuja grown herb for the purpose of approving the material for use in GMP production of CONAVIR®.

EXPERIMENTAL

Macroscopic and sensory examinations of the fresh plant

These were carried out on plants obtained from NIPRD's botanical gardens, as described by WHO (1998). The shape, size, color, odor and taste of the aerial parts were examined. Events and aspects related to the life cycle, habits and habitat were noted.

Treatment and sampling of the dried aerial parts

The aerial parts of herb harvested during the months of September and October were air-dried in a well ventilated shade in the Institute for drying medicinal plant materials and subsequently comminuted to coarse powder with a grinding machine. The procedure was as described by WHO (1998) as follows: Three original samples from each batch were combined into a pooled sample and subsequently used to prepare the average sample. The average sample was prepared by "quartering" the pooled sample as follows. Each pooled sample was mixed thoroughly, and constituted into a square-shaped heap. The heap was then divided diagonally into 4 equal parts. Any 2 diagonally opposite parts were taken and mixed carefully. This step was repeated as necessary until the required quantity of sample was obtained. Any material remaining was returned to the batch. The final samples were obtained from an average sample by quartering, as described above. This means

that an average sample gave rise to 4 final samples. Each final sample was divided into 2 portions. One portion was retained as reference material, while the other was tested in duplicate or triplicate.

Determination of bitterness value (BV)

The bitterness of the herb was determined by the method of WHO (1998) that compares the threshold bitter concentration (TBC) of an extract of the herb with the TBC of a dilute solution of quinine hydrochloride. The bitterness value is expressed in unit's equivalent to the bitterness of a solution containing 1 g of quinine hydrochloride in 2000 ml. The method is identical to that described in the European Pharmacopoeia (2006) as recently used by Meyer et al. (2009). The bitterness value is calculated as follows:

$$\text{Bitterness value in units per g} = (2000 \times C) / (A \times B)$$

where:

A = the concentration of the herbal stock solution (S_h) in (mg/ml).

B = the volume of S_h (in ml) in the tube with the threshold bitter concentration.

C = the quantity of quinine hydrochloride (in mg) in the tube with the threshold bitter concentration.

PHYSICOCHEMICAL TESTS

The following tests, briefly described, were carried out on the extracts as per WHO (1998):

Loss on drying (LOD)

This was carried out using a minimum of 0.5 - 1.0 g of material. Drying was effected in a gravity-convention oven (Lindberg/Blue M) maintained at 105-110°C. The results are expressed as a range or as mean \pm standard deviation. The LOD results were validated by concurrent determination of the LOD of CuSO₄ crystals, which was 36.43 %w/w.

Total ash (TA) and acid insoluble ash (AIA)

These values were determined using a minimum of 0.5 - 1.0 g of material and a furnace (Vecstar Furnace) heated gradually to the ignition temperature of 650 - 700°C. The process was repeated until at least two consecutive constant weights were obtained. The results are expressed as a range or as a mean value \pm standard deviation. The TA results were validated by concurrent determination of the TA of paracetamol BP, which was less than 0.01% w/w.

Water extractable matter (WEM) by hot extraction

About 4.0 g of coarsely powdered, air-dried sample is accurately transferred into a glass-stoppered, 250 ml, reflux conical flask, followed by 100 ml of water. The flask is weighed with the contents, and the weight is recorded (W_1). The flask is well shaken, and allowed to stand for 1 h. Subsequently a reflux condenser was attached to the flask, and boiled for 1 h; then the flask is cooled and weighed again with the contents - the weight (W_2) is recorded, and readjusted to (W_1) with water. The flask is again well shaken, and the contents rapidly filtered through a dry filter paper. By means a

pipette, 25.00 ml of the filtrate is transferred to a previously dried and tarred glass dish. The dish is then gently evaporated to dryness on a hot plate. Subsequently, the dish is dried at 105°C for 6 h, cooled in a desiccator for 30 min and weighed. The water extractable matter (WEM) is calculated as %w/w of the air-dried sample.

PHYTOCHEMICAL TESTS

The following tests as described by Harborne (1984) and Onwukaeme et al. (2007) were carried out on the herb or aqueous extract as follows:

Fehling's test for reducing sugars liberated by acid hydrolysis of glycosides

To about 10 mg of the extract in test-tube was added 2 ml of water, followed by 0.2 ml of 0.1 M HCl to effect hydrolysis of glycosides. In the control, 0.2 ml of water was used instead of the acid. The mixture was heated in a boiling water bath to accelerate dissolution, and thereafter left on the bath for 5 min longer. Subsequently, 1 ml each, of Fehling's solutions A and B, were added with shaking in the same bath for 10 min. A brick-red precipitate indicated the presence of reducing sugars, formed from the hydrolysis of glycosides.

Frothing test for saponins

A pinch (~ 50 mg) of the aqueous extract was added to 5 ml of water and warmed until dissolved. The solution was subsequently shaken vigorously to generate froth, and then allowed to stand. A rich froth persisting after 10 min indicated the presence of saponins.

Borntrager's test for anthraquinone derivatives

About 100 mg of air-dried herb was extracted with 5 ml of chloroform by shaking and warming under a bath. To about 2 ml of the supernatant, 1 ml of dilute 10% v/v ammonia solution was added, followed by shaking. A pink or red color in the aqueous layer is presumed to indicate the presence of anthraquinone derivatives.

Ferric chloride solution test for tannins

A pinch of the aqueous extract was vigorously shaken with 3 ml of warm water until dissolved. This was followed by 1 ml of 15 % ferric chloride test solution. A blue-green coloration indicated tannins.

Dragendorff's tests for alkaloids

The Dragendorff's test is a presumptive test for alkaloids. The color of precipitates produced in the test ranges from orange-brown to reddish-brown. The reagent consists of two solutions: Solution A: 1.7 g basic bismuth nitrate in 100 ml water/acetic acid (4:1); and Solution B: 40.0 g potassium iodide in 100 ml of water. The two are mixed as follows: 5 ml Solution A + 5 ml Solution B + 20 ml acetic acid + 70 ml water, to give 100 ml of the "Dragendorff's reagent". The test was carried as follows: About 20 mg of the herb was extracted with 20 ml of methanol by shaking and heating under a boiling water bath. The extract was subsequently filtered and allowed to cool. Each 2 ml of the filtrate in a test-tube was treated with 2 ml of the reagent. The development of an orange-brown precipitate is presumed to indicate the presence of alkaloids.

THIN LAYER CHROMATOGRAPHY (TLC)

Florescent, precoated plates were used for both the normal and reverse phase TLC. The normal phase utilized silica K6, and hexane: ethylacetate: methanol (4:4:1) as mobile phase; while the reverse phase utilized KC18 plate, and methanol: water (80:20). Solutions of analytes were prepared and applied as follows: To 1 mg of the analyte, 2 drops of ethanol were added and mixed well (~1% w/v solution). The plates used were 5 cm wide x 20 cm long. With a ruler and a pencil, a distance of 5 mm was measured from the bottom of the plate, and a line of origin was lightly drawn across the plate, without disturbing the adsorbent.

The analyte was applied to the origin as a 1 µl droplet. The spot was allowed to dry. Subsequently, the plate was developed in a developing tank saturated with the vapour of the solvent system to be used as mobile phase. The level of the solvent in the tank was adjusted to a level 2 to 3 mm below the line of origin on the plate. The plate was considered developed when the solvent front reached a predetermined line, not less than 5 mm below the top of the plate. The air-dried plate is visualized using a viewing cabinet (CAMMAG) and a UV-lamp (CAMMAG – equipped to emit light at 254 or 366 nm). The resulting chromatogram is photographed or drawn to scale.

RESULTS

The results of the macroscopic and sensory examination of the Nigerian grown *Andrographis paniculata* are shown Figure 1. The legend describes the key characteristics of the Nigerian herb, as compared to the Asian. The results of the determination of bitterness of the dried herb are presented in Table 1. The results show that men were more sensitive than women to the bitterness of quinine hydrochloride – the difference being significant at ($P < 0.05$)^a. But, although men appeared less sensitive to the bitterness of *Andrographis paniculata*, in that their mean bitterness value was lower than those of women, the difference was not significant ($P > 0.05$; two-tail, 10 degrees of freedom). Table 2 compares the Nigerian herb with the Asian, revealing physicochemical similarities, in terms of loss on drying; ash values; and water extractability. Table 3 shows that acid hydrolysis of the sample produced reducing sugars, implying the presence glycosides in the herb. The presence of saponins was revealed by the foaming tendency of the aqueous extract. Table 3 also suggests that detectable quantities of tannins and alkaloids, but not of anthraquinones, were present in the herb. Figure 2 depicts that normal phase TLC of the herb or its aqueous extract yielded 5 spots as against 6 spots yielded by reverse TLC.

DISCUSSION

GMP is the guiding principle for quality assured production. It has overtime attained its most eloquent expression in the food and drug industry. By law most countries require manufacturers of food and drugs to follow GMP procedures. However, GMP guidelines are not prescriptive instructions on how to manufacture, but

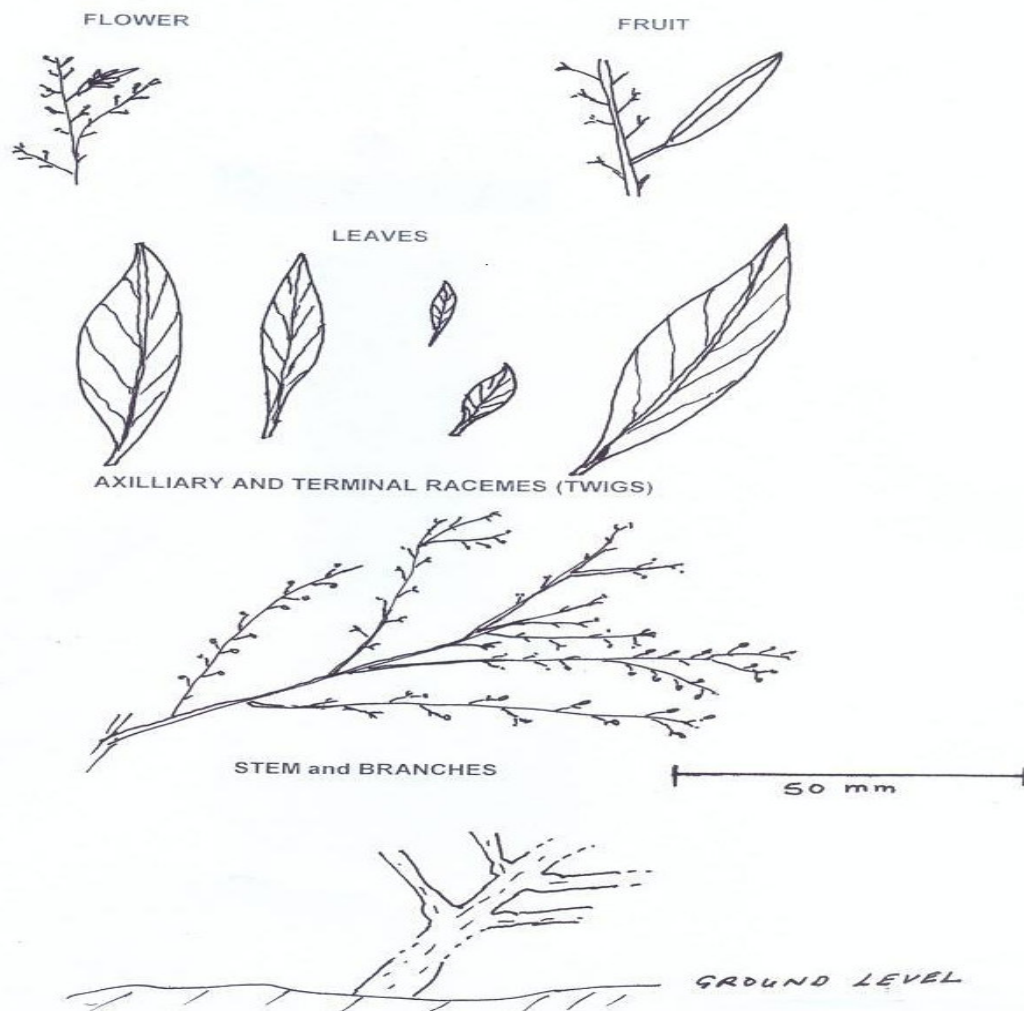
ILLUSTRATIONS OF AERIAL PARTS OF *ANDROGRAPHIS PANICULATA*

Figure 1. Aerial parts of *A. paniculata* with comments on habits and habitat. The flowers are tiny inflorescences with minute white petals bearing purplish spots; and occurring on lax spreading axillary and terminal racemes. The fruits bear numerous tiny brown seeds (less than 0.25 mm diameter) and occur in linear-oblong capsules that taper at the ends, measuring up to 2.0 cm long and 0.3 cm wide. The leaves are glabrous varying in shape and size as shown above; and have a prominent midrib from which arise four radiating veins. The mature leaves measure 3 - 9 cm long \times 0.7 - 2.0 cm wide. The herb is a perennial, mostly but not always grows erect to a height of 25 - 110 cm. The stem is deep green with diameter measuring 2 - 6 mm; is quadrangular with longitudinal furrows and wings at the axils of younger parts; and is slightly enlarged at the nodes. Each part above except the flowers and the seeds (which were not examined for taste and odor) are intensely bitter and odorless.

rather a series of principles that must be observed during production (Obodozie et al., 2009). Accordingly, the herbal material utilized in the production of CONAVIR[®] must meet the set of quality criteria prescribed by WHO (1998) for medicinal plant materials intended for production. The criteria include:

(1) The material must be authenticated botanically or histochemically, and must be obtained from an approved source.

(2) Features of the material such as its loss on drying (LOD), total ash (TA) and water extractable matter (WEM) must fall within $\bar{x} \pm 3SD$, where \bar{x} is the mean of up to 20 or more results, and SD is the standard deviation.

(3) Phytochemical tests and TLCs on the material should reveal a consistent phytochemical profile and fingerprints, respectively.

(4) Any pharmacological or biological test considered to be of particular interest, such as the determination of

Table 1. Determination of the bitterness value (BV) of *Andrographis paniculata*.

Volunteer (♂ or ♀)	[C] mg of S _q in tube with TBC	[A] mg/ml of S _h	[B] ml of S _h in tube with TBC	Bitterness value: units/g (2000 x C)/ (A x B)
SJA – male	0.046	0.01	6.0	1.53 × 10 ³
MJS – male	> 0.058 limit	0.01	-	-
NKO – male	0.052	0.01	> limit of 10	-
OOD – male	0.044	0.01	6.0	1.47 × 10 ³
ATA – male	0.050	0.01	7.0	1.43 × 10 ³
DAE – male	0.046	0.01	2.0	4.60 × 10 ³
CHS – male	0.046	0.01	7.0	1.31 × 10 ³
Mean ± SD	0.043 ± 0.003 ^a	-	-	2.07 ± 1.42 ^b × 10 ³
MOI – female	0.046	0.01	5.0	1.84 × 10 ³
OBS – female	0.044	0.01	4.0	2.60 × 10 ³
CSO – female	0.048	0.01	2.0	4.80 × 10 ³
RHB – female	0.054	0.01	2.0	5.40 × 10 ³
EOO* – female	0.052	0.01	8.0	1.30 × 10 ³
BSA – female	0.052	0.01	2.0	5.20 × 10 ³
Mean ± SD	0.049 ± 0.004 ^a	-	-	3.52 ± 1.82 ^b × 10 ³

(^a) indicates that the difference between the means is statistically significant at $P < 0.05$. This suggests that the males were more sensitive than females to the bitterness of quinine hydrochloride. Although the males appear less sensitive to the bitterness of *Andrographis paniculata*, in that the mean bitterness value for males appeared to be lower than that of the female, the difference denoted by (^a) is however not statistically significant at $P = 0.05$ (two-tail, 10 degrees of freedom). The mean bitterness value for both sexes is $2.86 \pm 1.74 \times 10^3$. The volunteer denoted (*), aged 42, had cold at the time of the experiment. If the result of the volunteer (1.30×10^3) is set aside, the female mean value becomes $3.97 \pm 1.63 \times 10^3$. Similarly, if the most extreme male result (4.60×10^3) is set aside, the male mean value becomes $1.44 \pm 0.09 \times 10^3$. In that scenario the sex difference denoted by (^c) becomes statistically significant at $P < 0.05$ (two-tail, 8 degrees of freedom). It may be stated that the large variation in the bitterness values is not unusual. For example Meyer et al (2009) had bitterness values of $58.1 \pm 110 \times 10^5$ and $51.6 \pm 156 \times 10^5$ for Praziquantel and (R)-Praziquantel, respectively.

Table 2. Physicochemical properties of aerial parts of *Andrographis paniculata*.

Parameter (%w/w)	Material from NIPRD	Material reported by WHO (2006)
Description	Dry, dark green aerial parts; practically odorless or faint and characteristic aroma.	-
Loss on drying	10.64 ± 0.36 (7)	Not more than 10% w/w
Total ash	14.10 ± 4.49 (7)	-
Water extractability	30.37 ± 2.63 (8)	Not less than 18% w/w
Acid insoluble ash	1.00 ± 0.06 (2)	Not more than 2% w/w

The numbers in parentheses indicate the numbers of samples/ determinations carried out in duplicates or triplicates.

Table 3. Inference from tests for phytochemical constituents of *Andrographis paniculata*.

Test	Observation	Inference
Fehling's test for glycosides	Red ppt. results	Glycosides are present
Frothing test for saponins	Copious froth results	Saponins are present
Test for anthraquinones	No apparent change	Anthraquinones may be absent
FeCl ₃ test for tannins	Blue-black ppt. results	Tannins are present
Test for alkaloids	Yellowish brown or orange ppt. results with Dragendorff's reagent	Alkaloids are present

The likely absence of anthraquinones in *Andrographis paniculata* is a favorable outcome since the material is intended for internal use.

bitterness in the case of *Andrographis paniculata*.

5) The material must pass the tests for arsenic/heavy

metals, microbial load, specific organisms (such as coliform bacteria or Salmonella) and aflatoxins; and if

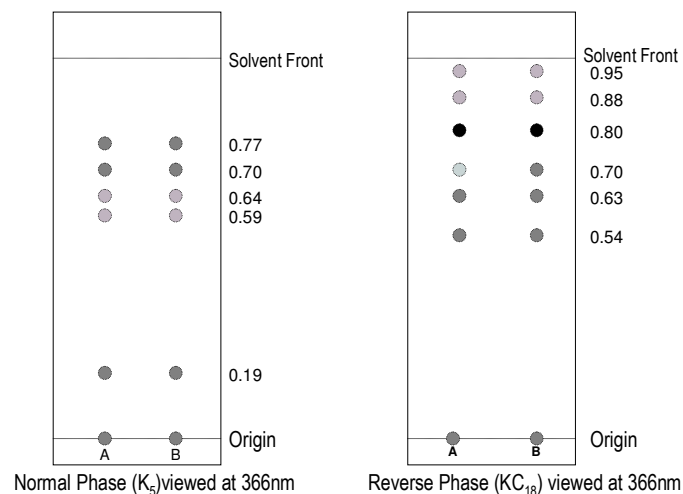


Figure 2. Diagrammatized TLC of ethanolic extract of *Andrographis paniculata*. A: Herb as ethanol extract; B: Herb as aqueous extract dissolved in ethanol. Mobile phase for the normal phase: Hexane: Ethylacetate: Methanol (4:4:1); Mobile phase for the Reverse Phase: Methanol: Water (80:20). The figures on the right of each plate are the Rf values of the spots on the left.

necessary, the tests for pesticides and radioactivity as well.

In Nigeria GMP is enforced by NAFDAC, whose approach, like those of most developing countries, is formulated on WHO guidelines (WHO, 1998; 2003). Ever since the Alma-ata Declaration of 1978, which heightened global interest in herbal medicines (Ameh et al, 2010), many countries today are inclined to apply GMPs even more rigorously to herbal preparations. For instance the US Food and Drug Administration will by June 2010 regard as “adulterated” any dietary supplements (as herbal products are called in the US) not produced in accordance with GMP. Perhaps the overriding paramountcy of GMP can be deduced from the fact that a product may be deemed by law to be “adulterated” even if there was no specific regulatory requirement violated in its production, so long as the process was not performed according to GMP (USFDA, 2007). It is in the light of the foregoing that the present report has relevance to CONAVIR[®]. The macroscopic and sensory characteristics of the fresh aerial parts of *Andrographis paniculata* are presented in Figure 1. The dried material from which the herbal component of CONAVIR[®] is derived, should be required to possess quality criteria akin those reported in this paper, as follows: BV, $2.86 \pm 1.74 \times 10^3$ units per g (Table 1); LOD, 10.64 ± 0.36 %w/w; TA, 14.10 ± 4.49 %w/w; AIA, 1.00 ± 0.06 %w/w; WEM, 30.37 ± 2.63 %w/w (Table 2). Table 2 also indicates key similarities between the Nigerian grown herb and the Asian, as reported by WHO (2006). The phytochemical and TLC studies should reveal a consistent profile and fingerprints similar to those shown

in Table 3 and Figure 2, respectively. These tests alone do not however, exhaust the WHO (1998) requirements, in that the toxicological parameters such as heavy metals, aflatoxins and pesticides are yet to be reported.

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