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QUALITY EVALUATION OF POZA BITTERS, A NEW POLY HERBAL FORMULATION IN THE NIGERIAN MARKET

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Abstract

Background: Development of a quality assurance system for new botanicals for health and nutrition is challenging. Producers must understand consumer demands and develop methods of production that meet those demands of product quality and efficacy, which exceeds or corresponds to international standards. The present article is an attempt to evaluate the quality of Poza bitters, a new polyherbal formulation, used as a purgative, by physical and analytical methods.
Materials and Methods: Poza bitters was screened for the presence of secondary metabolites and microbial contamination. The pH, micronutrient and heavy metal composition analysis, as well as chromatographic fingerprinting using thin layer chromatography and high performance liquid chromatography were also carried out.
Results: Phytochemical analysis revealed the presence of alkaloids, saponins, tannins, and flavonoid glycosides. The pH value was 3.66. The sample assayed had acceptable level of microorganisms and toxic metals were absent. Thin layer chromatography of the sample using methanol/water/acetic acid (30:70:5) showed three spots with R_f values similar to some of references used. High performance liquid chromatography fingerprint showed two retention times of poza bitters which were not similar to those of the reference standards: hesperidin and narigenin, thus suggesting the absence of these flavonoids in poza bitters.
Conclusion: Poza bitters comply with WHO microbial standard as well as standards for heavy metals. Also, the presence of Aloe ferox and citrus was confirmed on TLC.

Key words: Poza bitters, Quality evaluation, poly herbal, poza bitters.

Introduction

Herbal drugs are used as remedies for various diseases across the world from ancient time. In recent years, increasing interest has been focused on phyto-medicines as safer and more congenial to the human body. Medicinal plants come into preparation of various drugs singly or in combination or even are used as the principal source of raw material for the other medicines (Mohanta et al., 2003). An Herbal bitter which is a combination of bitter herbs is one of such herbal drugs being used for indigestion, weight control, detoxification and as antibacterial agents. Its long history and the belief that herbal products are natural and safe makes herbal bitters a ready alternative for medical conditions ranging from weight control, indigestion, tooth ache, insomnia to skin allergies.

For modern herbal products to be suitable for export and thus generate revenue for countries, standardisation of the raw materials to the finished products is necessary. Most of the regulatory guidelines and pharmacopoeias suggest macroscopic, microscopic evaluation and chemical profiling of the botanical materials for quality control and standardization (WHO, 1998). In this respect, protocols are based on most common parameters such as morphological evaluation, Physico-chemical evaluation, phytochemical screening and elemental analysis. Also, microbial contamination, test for specific pathogens such as *E. coli, Salmonella spp., S .aureus, Pseudomonas aeruginosa* are important. However, these parameters are judged subjectively and substitutes or adulterants which closely resemble the genuine material may be added. So chemical profiling is an essential parameter for standardization, which establishes a characteristic chemical pattern for a plant material, its fractions or extracts (Kartik Chandra Patra et al, 2010).

Poza bitters is a liquid formulation, which is used as a purgative. It is composed of *Aloe ferox (*21.25%w/v), *Citrus aurantifolia (*2.5%w/), and Honey (1.25%w/v). *Aloe ferox* is rich in anthraquinone glycosides: anthracene; barbaloin or aloin, isobarbaloin; aloinosides A and B used as a laxative and externally for *ulcus cruris*, eczema, burns, and in cosmetics (Agrawal and Paridhavi, 2007). *Citrus aurantifolia* is a pharmaceutical aid composed of flavonoid glycosides: hespiridin and naringin as well as volatile oils (Agrawal and Paridhavi , 2007) and used in the treatment of scurvy as well as for skin care, eye care, digestion, constipation, respiratory disorders, gout, weight loss, and urinary disorders due to presence of a large amount of vitamin-C and flavonoids, both of which are class-1 antioxidants, antibiotic and disinfectants (Agrawal and Paridhavi , 2007). Honey is used as an antibacterial, antioxidant, and in the treatment of a variety of ailments, from gastric disturbances to ulcers, wounds and burns. The biological activity of the formulation is related to the anthraquinone and flavonoid glycosides in its constituents.

In the present study, physical, chemical, and micobiological evaluation of poza bitters has been carried out because these evaluations are uncharted till date and determination of these parameters are very essential to assure the quality, safety, and efficacy of this formulation.

Materials and Methods

Determination of pH

Sample's pH was determined by standard method (Norris and Ribbon, 1970) using Bench-top pH meter (pH-016A model).

Qualitative phytochemical studies

Analysis for various phytoconstituents in the formulation was carried out using standard methods (Harborne, 1973). Presence of alkaloids, cardiac glycosides, tannins, flavonoids, and saponins were evaluated.

Elemental analysis

The atomic absorption spectrophotometer (Buck Scientific 210/211 VGP) run on acetylene gas was used for the detection of calcium,

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magnesium, potassium, sodium, manganese, iron, copper, zinc, chromium, cadmium, nickel, cobalt and lead.

Determination of Microorganism
Total viable aerobic count (TVC)

The TVC for aerobic bacteria and fungi (moulds and yeasts) were determined using plate count method after serial dilution. General purpose nutrient media and other selective media as appropriate were used for the culturing (Prescott et. al., 1999).

Tests for specific microorganisms

Test for specific organisms such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus*, Enterobacteriaceae and certain other gram negative bacteria such as *Escherichia coli* and *Salmonella* species were also done (Prescott *et. al.* 1999, Colle and Miles 1989).

Analytical TLC

TLC analysis of poza bitters was carried out using *Aloe barbadensis*, *Aloe ferox*, *Citrus aurantium*, hesperidin, and narigenin as references. Extracts of each sample was spotted on pre-coated TLC plate and developed in the different solvent systems. The R_f values were calculated for each spot. Mobile phase included different solvent systems such as Methanol: chloroform (7:3), Methanol: Ethyl acetate (8:2), Methanol: water (1:1), Methanol: water: acetic acid (30:70:5), and Methanol: Hexane (1:1). Detection was done in daylight, under UV at 254nm and 365nm and using vanillin- conc. sulphuric acid as spray reagent.

High Performance Liquid Chromatography (HPLC) Analysis

The HPLC analysis was performed on SESINADEPT SYSTEM 4. Column used was µBondapak C-18 (5µm, 150mm X 4.6mm) with injection volume and flow rate as 20µl and 1ml/min respectively. The mobile phase was 0.1% acetic acid: acetonitrile (80:20) and naringenin (0.0145g/ml), hesperidin (0.0136g/ml) were used as reference standards. The analysis was carried out at ambient (25°C) and detection was by UV spectrophotometer at 280nm

Results and Discussion

The pH was determined as a means of determining the gastric irritation potential of the preparation. The pH value was found to be 3.66 which is relatively safe to the gastric environment and can also prevent microbial spoilage of the formulation.

The results of the elemental analysis of poza bitters formulation are as presented in table 1. It included detection of micronutrients and heavy metals by means of atomic absorption spectrometry to ensure that the formulation has no constituent that could have deleterious effects on different organs of the body especially the kidney leading to renal toxicity. There are specified limits for each of these metals in herbal preparations. For lead, it should not be more than 2.0ppm. mercury ≤ 0.5ppm, cadmium ≤ 0.20ppm and aluminium ≤ 0.20ppm (Alwakeel, 2008; Ang, 2003 and 2006, Caldas et. al., 2004). There are also national limits specified by different countries. For example, Canada specified a maximum of 0.01mg/day for arsenic, 0.02mg/day for lead, chromium and mercury each and 0.06mg/day for cadmium while Malaysia specified 5mg/kg for arsenic, 10mg/kg for lead and 0.5mg/kg for mercury. WHO however recommends 10mg/kg for lead and 0.3mg/kg for cadmium (Patel et. al., 2011, Abbasi et al., 2010).

Of the 13 elements analysed, which included heavy metals like chromium, cadmium, nickel, cobalt and lead, 8 micronutrients which could be beneficial were found to be present in the sample while none of the heavy metals were present. Calcium had the highest concentration of 217.5mg/l and this is beneficial in promoting strong bones and teeth, and can prevent osteoporosis. Other micronutrients present are also essential in promoting adequate physiologic functions of the body. Iron is needed for blood cells, potassium for healthy nervous system and zinc for boosting immunity and performance of reproductive functions (Alwakeel, 2008). However, there are also maximum recommended doses allowed for these so called useful elements. Zinc should not exceed 5ppm while iron should not be more than 15ppm (Alwkeel, 2008). Sodium and potassium should also not be taken in excess to avoid hypertension as well as cardiac and metabolic problems. (Abbasi et al., 2010)

Table 1: Results of elemental analysis

Element	Conc.(mg/l)
Calcium	217.5 ± 0.16
Magnesium	84.9 ± 0.03
Potassium	65.8 ± 0.07
Sodium	66.1 ± 0.02
Manganese	6.21 ± 0.02
Iron	85.4 ± 0.06
Copper	0.098 ± 0.00
Zinc	0.040 ± 0.00
Chromium	0.00 ± 0.00
Cadmium	0.00 ± 0.00
Nickel	0.00 ±0.00
Cobalt	0.00 ±0.00
Lead	0.00 ±0.00

The results of microbiological screening of poza bitters are given in Tables 2 and 3. They included total viable count, detection of yeast and fungi, and specific bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Streptococcus* spp., *and Staphylococcus aureus.* The values obtained were within the limits specified by WHO for the microbial contamination in finished herbal products (Shrikuma et al., 2006). WHO

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specified that values for microbial limits should not exceed 10^5/g for total aerobic bacteria, 10^3/g for yeast and mould, 10/g for *E, coli* while *Salmonellae, Staphylococci* and *Pseudomonas* should be absent (Chitrarekha et. al., 2010, Mukherjee 2008).

Table 2: Total viable aerobic count

MICROORGANISMS	SAMPLE DILUTION	AVERAGE COLONY COUNT	
		48hours	72hours
Bacteria	10^{-5}	0	1
	10^{-6}	0	1
Yeast and fungi	10^{-5}	0	2
	10^{-6}	0	0

Table 3: Detection of specific microorganisms

MICRO ORGANISMS	SAMPLE DILUTION	AVERAGE COLONY COUNT	
		24 hours	48hours
Pseudomonas aeruginosa	10^{-5}	0	0
	10^{-6}	0	0
Staphylococcus aureus	10^{-5}	0	0
	10^{-6}	0	1
Streptococcus species	10^{-5}	0	3
	10^{-6}	0	1
Enterobacteriaceae	10^{-5}	0	0
	10^{-6}	0	0

The preparation therefore complies with microbial standard as prescribed by WHO.
The Phytochemical analysis results show the presence of alkaloids, saponins, flavonoids, tannins and deoxy- sugars. Anthraquinones, free flavonoids and unsaturated lactone were absent. Absence of unsaturated lactone but presence of deoxy sugar likely means that other glycosides which are not cardiac glycosides are present such as flavonoid glycosides. Alkaloids likely to be present include barbaloin from *aloe barbadensis*, The presence of these phytoconstituents in poza bitters is responsible for its usefulness as an antioxidant and as a laxative amongst many other uses. Flavonoids have antioxidant effect and also decrease capillary fragility (Ajibola and Motoyoshi 1992).

The results of TLC analysis are given in Table 4. Thin layer chromatography of poza bitters carried out to separate the various components using different solvent systems and reference samples. The reference samples *Citrus aurantium*, *Aloe barbadensis* and *Aloe ferox* were obtained from the Department of Pharmacognosy, University of Ibadan while Narigenin and Hesperidin were purchased from Sigma (USA). When viewed in the daylight and under UV (254nm and 365nm), a profile which was similar to the extract of *Aloe barbadensis* and *Aloe ferox* with similarities in the Rf values and colour intensity of the spots were observed. Compared to *citrus aurantium* and the pure flavonoids (narigenin and hesperidin; which are constituents of *Citrus* spp.), there was no similarity in the TLC profile. After spaying with vanillin- H_2SO_4, all the spots had relatively similar colours.

The number of spots obtained on the TLC plate varied with the different solvent systems used. Methanol/water/acetic acid (30:70:5) gave 4 spots for the sample (poza bitters) and for the *Aloe spp*. while the others gave less than 4 spots. This solvent system is therefore the best for resolution of the sample and references. The R_f of the spots of *Aloe ferox* and poza bitters were similar (0.34 and 0.57). This confirms the presence of Aloe ferox in poza bitters on TLC analysis. The presence of certain constituent of citrus (Rf 0.73) could be detected in Poza bitters. Reference flavonoids narigenin and hesperidin were however not qualitatively determined from the TLC since the Rf values obtained for poza bitters differ from those of narigenin and hesperidin.

Conclusion

WHO has emphasized the need to ensure quality control of herbal formulations by using modern techniques and by applying suitable parameters and standards (WHO, 2007). It is the cardinal responsibility of the regulatory authorities to ensure that the consumers get the medication, with purity, safety, potency, and efficacy. As prescribed by the WHO, evaluations of quality parameters (physical: pH ; chemical: HPLC, TLC and phytochemistry and microbiological) are essential to standardize the various herbal formulations. In conclusion, this study provides certain relevant standardisation parameters for the polyherbal formulation poza bitters. However, further work needs to be done to quantify the active constituents as well as assess the efficacy of the formulation.

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Table 4: Analytical TLC result for both the standard and references

S/N	Solvent	Sample	R_f Value	Colour in daylight	Colour in UV		Colour after spraying
					254nm	365nm	
1	Methanol: chloroform	A	0.86	Faint yellow	purple	white	Deep yellow/orange
		b	0.90	Yellow	Yellow	Red	Deep yellow/orange
		C	0.83	Yellow	Yellow	Red	Deep yellow
		D	0.79	Faint yellow	Yellow	Purple	Light yellow
		E	0.87	Faint Yellow	Purple	White	Light yellow
		F	0.70	Yellow	purple	Red	Deep yellow
2	Methanol: ethyl acetate	A	0.78	Faint yellow	purple	white	Yellow
		B	0.83	Yellow	Yellow	Red	Light brown
		C	0.85	Yellow	Yellow	Red	Yellow
		D	0.83	Faint yellow	Yellow	Purple	Orange
		E	0.85	Faint yellow	Purple	White	Light yellow
		F	0.74	Yellow	purple	Red	Yellow
3	Methanol :water	A	0.87	Light Yellow	purple	white	Yellow
		B	0.76 0.94	Brown	Yellow	Red	Light brown
		C	0.79 0.92	Brown	Yellow	Red	Yellow
		D	0.84	Colourless	Yellow	Purple	Light yellow
		E	0.82	Colourless	Purple	White	Light yellow
		F	0.77 0.89	Yellow/ brown	purple	Red	Yellow
4	Methanol: water: acetic acid	A	0.73 0.91	Colourless	purple	white	Yellow
		B	0.36 0.57 0.80 0.93	Brown, light yellow, orange	Yellow	Red	Yellow
		C	0.34 0.57 0.91	Brown, light yellow, orange	Yellow	Red	Yellow
		D	0.55	Colourless	Yellow	Purple	Orange
		E	0.71	Colourless	Purple	White	light yellow
		F	0.34 0.57 0.73 0.84	Brown, light yellow, colourless, orange	purple	Red	Yellow
5	Methanol: hexane	A	0.61	Light brown	purple	white	Yellow
		B	0.41 0.73	Brown	Yellow	Red	Light brown
		C	0.36 0.68	Brown/ light orange	Yellow	Red	Yellow
		D	0.73	colourless	Yellow	Purple	Orange
		E	0.78	colourless	Purple	White	Light yellow
		F	0.58	Brown/ light orange	purple	Red	Yellow

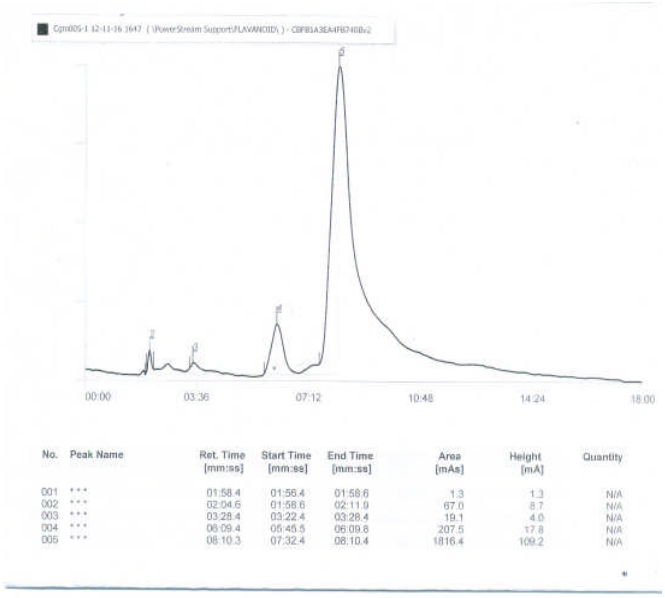
KEYS: *Citrus aurantium* (A), *Aloe barbadensis* (B), *Aloe ferox* (C), Narigenin (D), Hesperidin (E), Poza bitters (F).

The result of HPLC analysis of poza bitters, comparing it with two standards: hesperidin and narigenin. is given in table 5. The result showed relatively different chromatograms but close retention times were obtained at certain times in the analysis for both the sample and the standard such as retention times of 01:46.9 and 01:44.7 for poza bitters and narigenin respectively; and 03:26.3 and 03:28.4 for poza bitters and hesperidin respectively. The contents of narigenin and hesperidin however could not be quantified. Other retention times obtained imply other unknown constituents of poza bitters.

Table 5: HPLC Result

	No of peaks	Retention time (mm:ss)
Poza bitters	6	01:46.9, 02:12.8, 03:26.3, 04:05.9, 04:21.0,05:49.0
Hesperidin	5	01:58.4, 02:04.6, 03:28.4, 06:09.4, 08:10.3
Narigenin	6	01:43.6, 01:44.7, 01:52.0, 01:58.4, 02:08.4, 02:22.0, 13:47.1

http://dx.doi.org/10.4314/ajtcam.v12i1.3



No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]	Quantity
001	***	01:58.4	01:56.4	01:58.6	1.3	1.3	N/A
002	***	02:04.6	01:58.6	02:11.9	67.0	8.7	N/A
003	***	03:28.4	03:22.4	03:28.4	19.1	4.6	N/A
004	***	06:09.4	05:45.5	06:09.8	207.5	17.8	N/A
005	***	08:10.3	07:32.4	08:10.4	1816.4	109.2	N/A

a. Hesperidin

No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]	Quantity
001	***	01:43.6	01:42.8	01:43.9	0.7	1.2	N/A
002	***	01:44.7	01:43.9	01:45.1	1.8	2.1	N/A
003	***	01:52.0	01:48.4	01:54.6	232.1	79.2	N/A
004	***	01:58.4	01:54.6	02:03.4	311.8	55.2	N/A
005	***	02:08.4	02:03.4	02:13.8	295.2	34.8	N/A
006	***	02:22.0	02:14.0	02:41.4	1035.0	64.5	N/A
007	***	13:47.1	13:14.6	13:47.2	1328.7	96.5	N/A

b. Narigenin

No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]	Quantity
001	***	02:13.1	01:48.6	02:40.2	12796.9	972.9	N/A
002	***	03:25.9	03:11.9	03:26.1	456.1	46.1	N/A
003	***	04:06.3	03:48.8	04:06.6	575.1	51.0	N/A
004	***	04:21.2	04:11.2	05:03.4	1886.8	66.6	N/A
005	***	05:48.6	05:06.9	06:40.8	20822.9	761.1	N/A

c. Poza bitters

Figure 1: HPLC fingerprint of reference samples Hesperidin (a), Narigenin (b) and Poza bitters (c)

http://dx.doi.org/10.4314/ajtcam.v12i1.3

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