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Research Article

Keywords: Markers, phytochemical standardization, quality control, herbal materials, herbal products

Posted Date: April 4th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2735549/v1>

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Additional Declarations: No competing interests reported.

Application of the Herbal chemical Marker Ranking System (Herb MaRS) to standardization of herbal pharmaceutical raw materials-a case study.

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Abstract

Introduction

Phytochemical standardization of herbal materials involves establishing consistent levels of one or more active ingredients or markers. It ensures the authenticity and quality of herbal materials, extracts, and medicinal products. We successfully applied the Herbal chemical Markers ranking system (Herb MaRS) originally proposed for quality assurance of complex herbal products, to establish markers for controlling the quality of herbal raw materials. We used the most commercially consumed plant materials in Uganda, as a case study.

Methods

The assessment of compounds for suitability as markers were based on the Herb MaRS, with minor modifications as follows: For more objective scoring, evidence of biological activity of the potential marker compound (s) was determined at three levels based on the number of symptoms of the disease condition a compound can treat or alleviate; (i) one symptom (1 point), two symptoms (2 points), 3 and more symptoms (3 points). We also scored the reported concentrations of the compounds (concentration not determined (0 points), concentration ≥ 5 ppm (1 point), (concentration ≥ 50 ppm, 2 points) and availability of analytical standards (1 point); lastly, we scored the compounds for the availability of an analytical method (1 point). Compounds scored from 0 to 8; where 8 indicated the most suitable chemical marker.

Results

The selected markers were as follows: *Eucalyptus globulus* (aromadendrine, α -terpineol, globulol, and 1-8, cineol); *Aloe barbadensis* (aloin, aloe emodin, acemannan), *Albizia coriaria* (lupeol, lupenone, betulinic acid, betulin, and catechin); *Mangifera indica* (mangiferin, catechin, quercetin, and gallic acid); *Warburgia ugandensis* (polygodial); *Azadirachta indica* (azadirachtin, nimbin, nimbidin); and *Zingiber officinalis* (6, 8, 10-gingerols, and 6-shogaol).

Conclusions

Herb MaRS can be efficiently applied to select marker compounds for quality control of herbal materials. The markers identified should be incorporated into chromatographic fingerprints as well as quantitative methods and evaluated for applicability at the various stages of the production chain of herbal medicines. There is a need to build local capacity to isolate marker compounds, particularly those that are not sold by current vendors.

Keywords: Markers, phytochemical standardization, quality control, herbal materials, herbal products.

Introduction

Herbal materials vary greatly in chemical composition due to several factors including climate, cultivation, and harvesting practices, as well genetic differences among cultivars of the same species (1). To cater for this variability, herbal raw materials must be standardized before manufacturing medicinal products. Standardization involves activities that ensure that the materials and the resultant extracts are phytoequivalent. This ensures the reproducibility of efficacy and safety of the materials and their products (1, 2).

The evaluation of chemical constituents of plant raw material involves screening and quantification of the major phytochemical groups, the establishment of fingerprint profiles, and/or quantification of selected markers as shown in the scheme in figure 1.

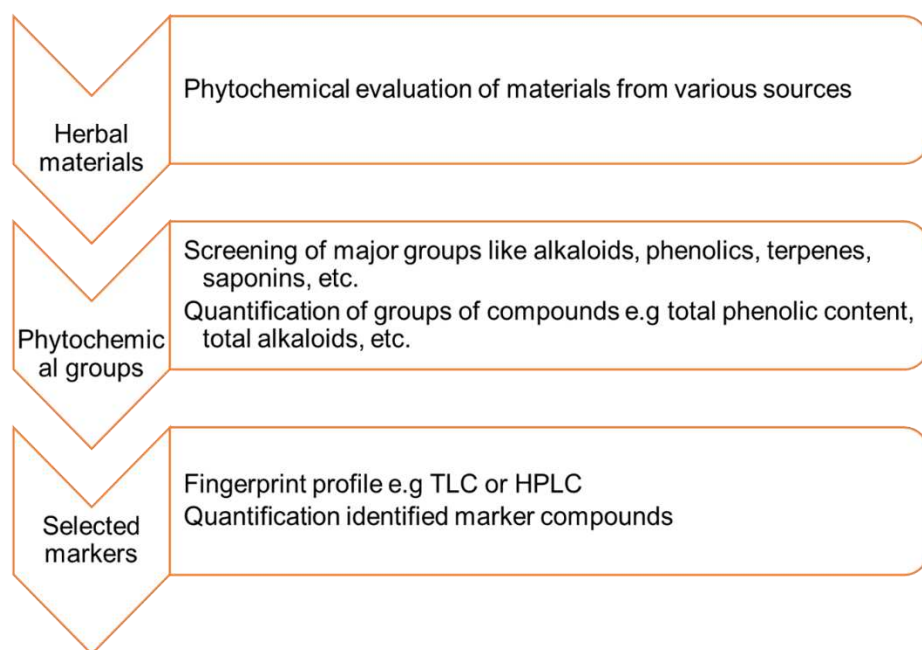


Figure 1: Phytochemical evaluation of herbal raw materials

Markers are measured in both raw materials and finished products. The information obtained from measuring markers can be used for several purposes. These include identification and selection of raw materials in cases where concentration limits are set, identification of adulterants and toxicants, assessing batch-to-batch uniformity of materials from different sources, controlling of the manufacturing process, assessing the suitability of packaging and storage, standardizing physiological activities; and calculation of dosage of raw materials to include in the product formula (2-4); as summarized in figure 2.

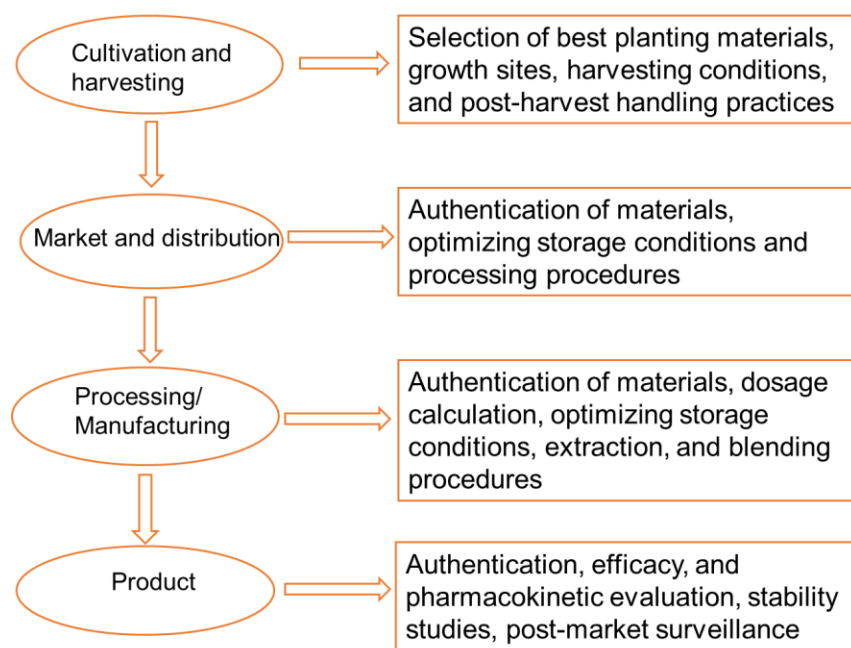


Figure 2: Applications of markers at different stages of the herbal medicines production chain

Selection of marker compounds

When choosing a marker(s) for routine quality control of herbal materials, the following factors should be considered: (i) local availability of effective and easy to use analytical methods such thin layer chromatography, high performance chromatography, and spectroscopy (ii) availability of analytical standards of acceptable quality, (iii) relevance of the compounds to therapeutic

application of the herbal material, and (iv) suitability of the compound(s) as stability indicators. According to the WHO, constituents with known biological activity (related to the traditional use of the herbal material), if known and available, should preferably be selected as markers. Otherwise, compounds with recognized biological activities or characteristic constituents can be used (3). In line with the WHO and other regulatory guidelines, the National Institute of Complementary Medicine, University of Western Sydney, has published a Herbal Chemical Marker Ranking System (Herb MaRS) for selecting markers for quality control of complex herbal products. Phytochemicals are ranked on a scale of 0 to 5 based on their concentration in the plant or product, evidence of efficacy relevant to the traditional use of the product, bioavailability and toxicity. Compounds for which there is no evidence of biological activity are ranked X (5).

This work aimed at establishing chemical markers for the seven most commonly used plant species in the manufacture of herbal medicinal products in Uganda, as established in our previous study (6). The study established that *Eucalyptus globulus* oil was by far the most used material, (in 50% of the products studied); *Aloe barbadensis* and *Albizia coriaria* were the second most commonly used, while, *Mangifera indica*, *Warburgia ugandensis*, *Azadirachta indica* and *Zingiber officinalis* tied in third place (figure 3) We considered these seven most used species and reviewed literature to identify the most suitable markers for their quality control.

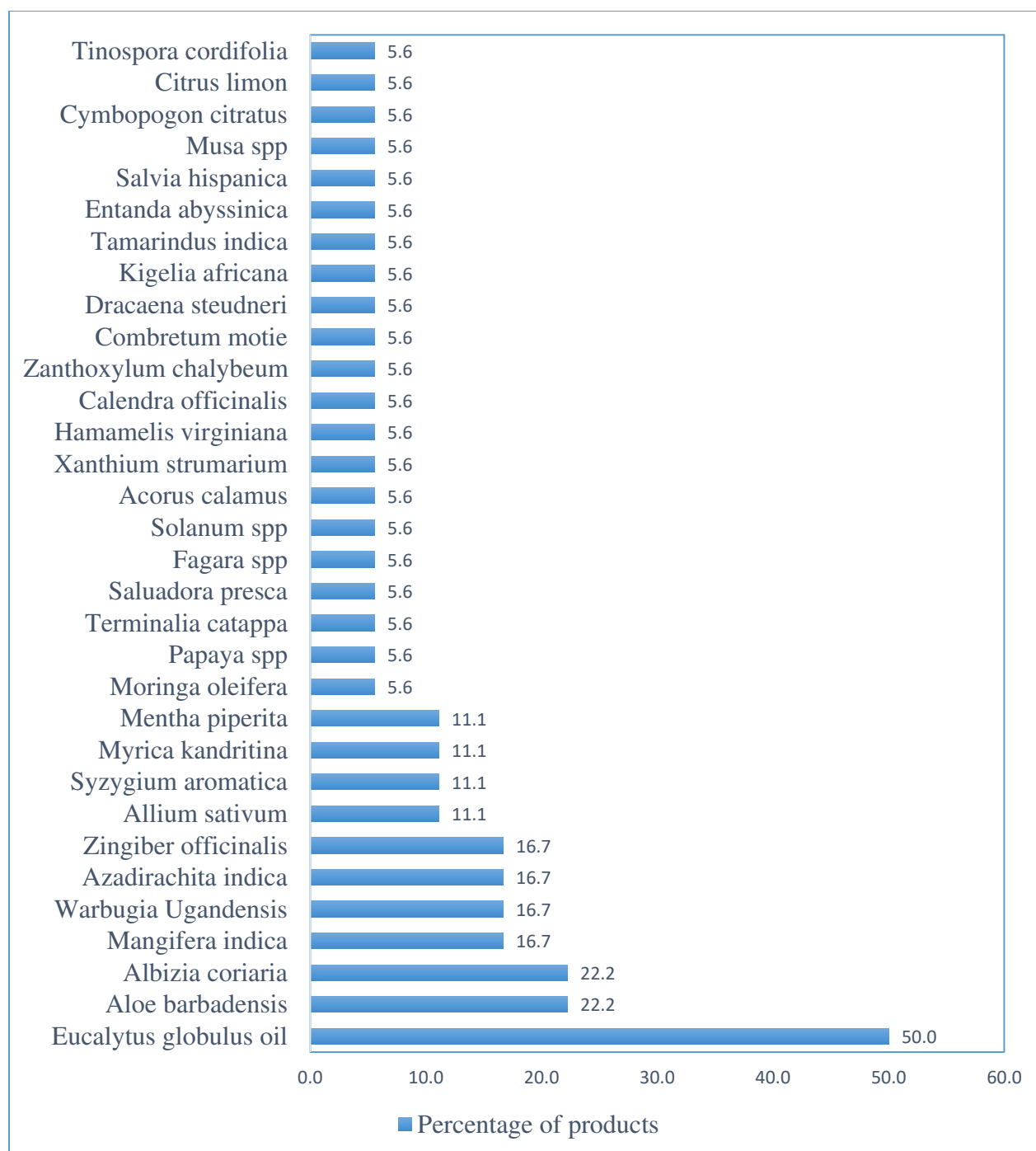


Figure 3: Frequency of use of herbal materials in Ugandan registered herbal products according to the 2018 National Drug Register; modified with permission from (6)

Methods

Identification of potential marker compounds

The suitability of analytical markers was determined based on the WHO guidelines for selecting marker substances of herbal origin for quality control of herbal medicines (3) and also on the Herbal Chemical Marker Ranking System (Herb MaRS) (5). Both the WHO guidelines and the Herb MaRS give priority to a compound whose biological activity is related to the traditional use of the plant and can be identified and quantified by the analytical methods available. Also, the compound chosen should be available commercially in pure form.

Establishment of active compounds and evidence of biological activity

Once the most consumed herbal materials were established, an extensive literature search to identify the bioactive compounds and compile the evidence of their biological activity relevant to the industrial application of the plant materials ensued. Such evidence included studies reporting on the ability of the compound (s) to treat or ameliorate one or more symptoms of the disease condition as indicated by the manufacturer. According to Kaggwa et al. (6), *Albizia coriaria*, *Mangifera indica*, and *Zingiber officinale* are exclusively used in cough syrups; *Warburgia ugandensis* in cough and anti-ulcer syrups; *Eucalyptus globulus* in mouth wash's, cough syrups and pain balms; *Aloe vera* in mouth wash's, cough syrups, lip balms and GIT cleansing tablets, *Azadirachta indica* in cough and anti-ulcer syrups as well as lip balms. The same study were established evidence of efficacy and safety of the plant materials for these industrial applications.

Table 1 summarizes the diseases (or their symptoms) the products are indicated to manage.

Table 1: Disease conditions managed by the most industrially consumed herbal materials in Uganda. Table modified with permission from (6)

No.	Plant material	Dosage form of product containing the material	Indication(s) of the products containing the material
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1	<i>Eucalyptus globulus</i>	Mouth wash	Toothache, bad odor, sensitivity, bleeding gums, (tooth) cavities, tooth decay, antibacterial, mouth sores
		Cough syrups	Cough, common cold, catarrh, sore throat, congestion from asthma, bronchitis, whooping cough, allergic conditions-sinusitis, rhinitis, mouth sores, hiccups, relieving fever, measles symptoms
		Pain balm	Pain relief
2	<i>Aloe vera</i>	Mouth wash	Toothache, bad odor, sensitivity, bleeding gums, (tooth) cavities, tooth decay, antibacterial,
		Aloe tablets	Cleanses GIT
		cough syrup	Cough, flu, sore throat, sinusitis
		Lip balm	Dry, cracked, and painful lips
3	<i>Albizia coriaria</i>	(Cough) syrups	Whooping cough, catarrh, sore throat, congestion from asthma and bronchitis, relieving fever, sinusitis
4	<i>Mangifera indica</i>	(Cough) syrups	Whooping cough, catarrh, sore throat, congestion from asthma and bronchitis
5	<i>Warburgia ugandensis</i>	(Cough) syrups	Cough, flu, mouth sores, measles symptoms, common colds, sinusitis, rhinitis, asthma, catarrh, whooping cough, bronchial congestion, mouth sores, hiccups
		(antiulcer) syrups	(GIT) Ulcers
6	<i>Azadirachta indica</i>	Cough syrup	Cough, flu, sore throat, sinusitis
		(antiulcer) syrups	Gastric ulcers, stomach ulcers, flatulence, constipation
		Lip balm	Dry, cracked, and painful lips
7	<i>Zingiber officinale</i>	cough syrups	Allergic cough, smokers cough, whooping cough, productive cough, flu, lung cleaning, sore throat, sinusitis, bronchial asthma, relieving fever

We browsed search engines like Google, and indexes including PubMed, Google Scholar, Researchgate, and Web of Science. The search terms consisted of the chemical name, pharmacological or therapeutic activity of interest; such as “anti-inflammatory activity of 6-gingerol”; plant botanical name and bioactive compounds thereof, such as; “bioactive compounds

in *Mangifera indica* leaves”, “mechanism of action of mangiferin” and so on. Only full-length articles published in English were reviewed.

Availability of analytical methods for the potential marker compounds

In addition to evidence of biological activity of the identified compounds, we also searched for information regarding quality control methods recommended by existing pharmacopoeial monographs, particularly the WHO monographs on selected medicinal plants (7-11), the African Pharmacopoeia, the West African Herbal Pharmacopoeia(12) and the Pharmaceutical monographs for South African plants species (13). Further still, we searched for availability of assay methods for quantification of markers in the respective plant materials. We focused on high performance liquid chromatography (HPLC) methods because they are highly sensitive, specific, and versatile. Where HPLC methods were not available or not suitable such as for essential oils, high performance thin layer chromatography (HPTLC), gas chromatography (GC) or ultra violet/visible (UV/VIS) spectrophotometric methods were considered.

Availability of analytical reference standards for the potential marker compounds

We assessed for the availability of analytical standards and the prices for the smallest units. For this we preferably searched the Sigma Aldrich ® website (<https://www.sigmaaldrich.com/UG/en>) for two main reasons: (i) from our experience, they are the most reliable supplier of high-quality chemicals, and (ii) they willingly display the prices for various grades and quantities of the same analytical standard. If the compounds were not available from Sigma Aldrich ®, we did a general google such to establish other potential sources. Finally, the cost of 1mg or mL of the marker was computed with an assumption this amount is sufficient for a single analysis-to construct calibrations curves.

Selection of the most suitable marker compounds

Using a modified Herb MaRS, compounds were given scores from 0 to 8; where 8 indicates the most suitable chemical marker. The Herb MaRS (see details here (5)) does not elaborate on how the individual attributes of the compound are scored but rather gives a lumpsum mark after the overall assessment. Therefore, for more objective scoring, we modified the ranking system as follows: Evidence of biological was divided into three levels based on the number of symptoms of the disease condition a compound can treat or alleviate; (i) one symptom (1 point), two symptoms (2 points), 3 and more symptoms, with well elucidated mechanisms of action (3 points). We also scored the reported concentrations of the compounds (concentration not determined (0 points), concentration ≥ 5 ppm (1 point), (concentration ≥ 50 ppm, 2 points) and availability of analytical standards (1 point); lastly, we scored for availability of an analytical method (1 point).

Results

Evidence of biological activity of potential chemical markers

For most of the plant materials, there are compounds with biological evidence relevant to the industrial application of the products in which the medicinal plant is contained. However, most of the compounds in *Warburgia ugandensis* have not been individually evaluated. The results are summarized in Table 2.

Table 2: Biological evidence of typical compounds found in the plant materials

Plant species and manufacturer therapeutic claim	Bio-active compound	Evidence of Biological activity of bioactive compound related to traditional use of the plant	Mechanism of action
<i>Eucalyptus globulus</i> pain relief, URT disorders, mouth wash, GIT disorders	Aromadendrine (dihydrokaempferol)	Anti-inflammatory Anti-oxidant	Scavenging of reactive oxygen species, chelation of metal ions(14)
		Antibacterial; <i>Staphylococcus aureus</i> , <i>Streptococcus mutans</i>	Inhibits biofilm formation(15)
	1,8-cineol	Antibacterial-against <i>K. pneumoniae</i> , <i>S. aureus</i> , <i>P. aeruginosa</i>	Disruption of bacterial cell membrane & loss of intracellular materials (16)
		Antiasthma, anti-bronchitis, anti COPD, Anti-influenza	Down-regulation of inflammation cytokines such as interleukin- 1b (IL-1b) and tumor necrosis factor- α resulting in bronchial muscle relaxant and reduction in mucus secretion (17)
		Anti-inflammatory Anti-oxidant	Regulates nuclear factor-kappa B (NF- κ B) and nuclear factor erythroid-2-related factor 2 (Nrf2) pathways(18, 19)
		Analgesic/sedative	Suppression on the CNS by modulating glutamatergic & dopaminergic systems, activates transient receptor potential melastatin 8 (20, 21)
	globulol	Antispasmodic and antisecretory, gastroprotective, antidiarrheal	Promotes regeneration of the gastric cells, increases gastric mucus, antioxidant anti-inflammatory effects (18)
		Antibacterial-against <i>K. pneumoniae</i> , <i>S. aureus</i> , <i>P. aeruginosa</i> (22, 23)	

	α -terpineol	Anti-inflammatory & antioxidant mainly analgesic	Suppresses superoxide production by monocytes; inhibits release of inflammatory mediators including serotonin, histamine, bradykinin, & prostaglandins (24, 25)
		Antibacterial-against <i>K. pneumoniae</i> , <i>S. aureus</i> , <i>P. aeruginosa</i>	As 1,8-cineole
		Anti-gastric ulcer	As 1,8-cineole
<i>Aloe barbadensis</i> cracked lips, mouth wash, URT disorders, GIT disorders	Aloin A and B Aloe emodin	Laxative	Inhibition of Na ⁺ /K ⁺ pump & Cl ⁻ channels increase gastric motility; stimulate secretion of mucus and chloride ions (26, 27)
		and immune-modulatory effects	Inhibition of histamine release from mast cells -reduced production of TNF- α (28)
		anti-inflammatory- anti-oxidant	Cyclooxygenase pathways and reducing prostaglandin E2 production (29, 30)
	Acemannan	anti-bacterial activity-H pylori	Activation of phagocytic leukocytes; inhibition of the N-acetyltransferase activity of <i>H. pylori</i> (31, 32)
		Wound healing anti-inflammatory	Activates macrophages to release fibrogenic cytokines; inhibits thromboxane A ₂ (33, 34) Cyclooxygenase pathways and reducing prostaglandin E2 production(28)
	Mannose phosphate	6 Wound healing	Enhances activity of fibroblasts (35, 36)

	Lupeol		Anti-inflammatory- anti-oxidant	See for below <i>Albizia coriaria</i>
<i>Albizia coriaria</i> URT disorders,	Lupeol lupenone	and	Anti-inflammatory Analgesic	Decreases PGE ₂ , TNF- α , interleukin-1b production (37) (38)
			Immunomodulating; anti allergen, anti- asthmatic,	Reduces the production eosinophils, thus interleukins-reduced fluid production in the bronchoalveolar pathway (39)
			Antibacterial-against <i>S. aureus</i> , <i>K. pneumoniae</i> , <i>P. aeruginosa</i> (40, 41) Anti-viral-herpes simplex(42)	
	Betulinic acid/betulin		Anti-inflammatory Analgesic	Inhibits production of nitric oxide & PG ₂ (cyclooxygenase-2 activity); also decreases production of pro-inflammatory cytokines including IL-1 β , IL-6, IL-8, IL-12, & TNF (43, 44)
			Antibacterial- <i>P. aeruginosa</i> , <i>S. aureus</i> , <i>Mycobacterium tuberculosis</i> (45)	
			Anti-viral-against herpes simplex(46)	
	(±) Catechin		Anti-inflammatory	Radical scavenging; activates production of erythroid-derived factor 2 which regulates antioxidant enzymes (47)
			Immunomodulatory; anti-allergenic	Reduces production & infiltration of lung tissue by inflammatory cytokines such as TNF- α , IL-1 β (48)
			Anti-viral-against Influenza A, SARS- CoV-2 (49, 50)	
<i>Mangifera indica</i> URT disorders,	Mangiferin		Antioxidant, anti-inflammatory, antipyretic, analgesic,	Scavenging of ROS, chelation of toxic metal ions; down-regulates phosphorylation of NF- κ B pathways-reduces production of proinflammatory cells(51, 52)

	Antiallergic, immunomodulatory	anti-asthmatic,	Reduces tracheal contraction by inhibiting the nitric oxide-cyclic GMP pathway(53); Inhibits production of nitric oxide and PG2 (cyclooxygenase-2 activity); also decreases production of pro-inflammatory cytokines including(54)
		Antibacterial-activity against <i>S. aureus</i> (55)	Increased antibody titers; increases cell mediated immunity. Stimulates lysozyme activity, (56)
		Antiviral-against Herpes simplex (57)	
(+/-) Catechin, epicatechin	Anti-viral, anti-inflammatory anti-allergenic		As for <i>Albizia coriaria</i> above
Gallic acid	Antioxidant, anti-inflammatory		Scavenging of ROS, chelation of toxic metal ions; down-regulates phosphorylation of NF- κB pathways-reduces production of proinflammatory cells [50-52]
	Antimicrobial- <i>aureus</i>	<i>P. aeruginosa</i> , <i>S.</i>	Interferes with colonization by inhibiting motility & adherence; disrupts cell membrane leading to leakage of cell nutrients; inhibits dihydrofolate reductase, topoisomerase IV (58, 59)
		Antiviral - <i>Haemophilus</i> influenza A & B	Disruption of the viral particles(60)
Quercetin	Antioxidant, anti-inflammatory,		Scavenging of ROS, chelation of toxic metal ions; down-regulates phosphorylation of NF-κB pathways-reduces production of proinflammatory cells, inhibits cyclooxygenase & lipoxxygenase enzymes (61-64)
	Immune-modulatory; anti-allergic		Inhibits IL 8 & 6, TNF-α

<i>Azadirachita indica</i> URT disorders, cracked lips	Tetranortriterpe- nes- Azadirachtin	Antibacterial; <i>P. aeruginosa</i> , <i>S. aureus</i> , <i>B. subtilis</i> , <i>M. tuberculosis</i> , <i>K. pneumoniae</i>	Inhibits nucleic acid synthesis, disrupts plasma membrane, inhibits glutamine synthetase, inhibits biofilm formation (65, 66)
		Antiviral - <i>Influenza-A</i> virus (67)	
	Nimbidin, Nimbin,	Anti-inflammatory, antipyretic, antioxidant Wound healing, Anti-gastric ulcer	Inhibits cyclooxygenase (COX), & lipoxygenase (LOX) enzymes. Modulates transcription factors NF-κB, radical scavenging
		Immunostimulant	Inhibits TNF-induced biological responses (68)
		Antibacterial- against <i>S. aureus</i> & MRSA	Inhibits biofilm formation (69)
	Nimbolide	Antipyretic, anti-inflammatory and antioxidant (70, 71)	Suppresses production of inflammatory cytokines from neutrophils & macrophages (72)
		Anti-gastric ulcer	Reduces secretion of gastric acid by inhibiting histamine (H2) receptors and muscarinic receptors (73)
		Immunomodulatory; anti-allergic	Inhibits macrophage migration (74)
	Mahmoodin	Antibacterial (75)	
	Diterpens- Margolone, margolonone, margolonone	Antibacterial-against <i>Klebsiella</i> , <i>Staphylococcus</i> & <i>Serratia</i> Species (76)	

	Gallic acids	Anti-inflammatory, immunomodulatory As described for <i>Mangifera indica</i>	Inhibits oxidative burst of polymorphonuclear cells during inflammation
<i>Zingiber officinalis</i> URT disorders, GIT disorders	Gingerols	Antioxidant activity,	Scavenging of ROS, chelation of metal ions;
	8- gingerol	Anti-inflammatory, analgesic: 6-	oxygenation arachidonic acid, a substrate for
	10-gingerol	gnigerol and 6-shogaol most studied	cyclooxygenase enzymes, thus inhibiting
	12-gingerol		production of prostaglandins; reduced
	6-gingerol		activation of macrophages; inhibit nitrite
	Shogaols		oxide (NO) production (77, 78)
	6-shogaol	Anti-asthmatic, anti-allergen	Reduced contraction of smooth respiratory muscles by reduction in Ca ²⁺ influx & β 2 receptor activation; reduced production of proinflammatory cytokines(79)
		Antibacterial- <i>S. aureus</i> , <i>Mycobacteria</i> , <i>Streptococcus pyogenes</i> , <i>Streptococcus pneumoniae</i> , <i>Hemophilus influenzae</i>	Inhibition of biofilm formation, inhibition of hydroxymethyl-7, 8- dihydro pterin pyrophosphokinase, 6-gingerol>8-gingerol>10-gingerol>12-gingerol (80, 81)
		Anti-gastric-ulcer activities -anti- <i>H. pylori</i> , 10-gingerol>8-gingerol>6-gingerol>6-shogaol(82) -anti-emetic-	Inhibit 5-hydroxytryptamine to increase gastric motility and emptying (83, 84)
	Zingerone (major pungent compound in ginger)	Anti-inflammatory antioxidant activity Antibacterial activity	Reducing ROS production; chelation of metal ions (85) Dihydro pterin pyrophosphokinase inhibition
<i>Warburgia ugandensis</i>		Anti-inflammatory and anti-allergic – polygodial	inhibition of phospholipase A2 and neuropeptide release (86)

URT disorders,	Drimane	Antimicrobial-antimycobacterial
	sesquiterpenes	(muzigadial & muzigadiolide) (87)
	Muzigadial,	Most of the compounds have not been
	Muzigadiolide	elucidated for biological corresponding
	Warbuganal,	to traditional use of the herbal material
	warbugadione,	
	warbugadial,	
	warbugin	
	Ugandensidial,	
	Ugandensolide	
	polygodial	

Availability of analytical standards and assay methods

For *Eucalyptus globulus*, *Aloe barbadensis*, *Zingiber officinalis*, and *Azadirachta indica*, monographs with well elaborated quality control methods have been published. In addition, analytical standards for the selected compounds are available and their assay methods have been developed. On the other hand, no monographs for *Albizia coriaria*, *Mangifera indica*, and *Warburghia ugandensis* exist., for *W. ugandensis*, there are even no analytical standards or assay methods to quantify the individual compounds. The cost per mg or mL of analytical standard ranged from €0.6 to 498 for α -terpineol and azadirachtin respectively, with an average cost of €62.5±101.5. The results are summarized in Table 3.

Table 3: Availability analytical standards and assay methods for selected medicinal plant compounds

Plant material	Identified Analytical markers	Commercial sources	Smallest commercial unit available, purity	Cost per mg of marker	Analytical methods for the plant	Assay methods already developed for the compounds
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			and /cost (Euros- €)	(a single analysis)	material mentioned in local or WHO Pharmacopoeia	in same or other materials
<i>Eucalyptus globulus</i> oil	Aromadendrine (dihydrokaempferol)	Sigma- Aldrich/Supe lco	Analytical standard-530/5mg ≥95% HPLC grade- 221/10mg ≥95% LC/MS- ELSD-421/1mg	106.0 22.1 421	WHO monographs &African pharmacopoeia- identification tests for 1,8-cineole in oil & leaf materials. TLC fingerprint for leaf materials with 1,8-cineole as reference (8, 11).	Gas chromatography FID & MS method for quantification of oil components (23, 88)
	α-terpeneol	Sigma- Aldrich/Supe lco	Analytical standard (≥95%)- 63.9/100mg	0.6		
	globulol	Sigma- Aldrich	≥98.5% (sum of enantiomers, GC)- 247/100mg	2.5		
	1,8-cineol (eucalyptol)	Sigma- Aldrich/Supe lco	Analytical standard- 48.3/1mL	48.3		
Aloes - whole leaf products	Aloin A	Sigma- Aldrich	Analytical standard- 440/10mg	44.0	WHO monographs- Thin-layer chromatography, microchemical analyses to identify anthracenes, Spectrophotometry- to determine total glycosides as aloin (8)	HPLC-DAD/MS quantification methods for anthracenes in aloe vera samples (89)
	Aloin B	Sigma- Aldrich	Phyproof® Reference Substance- 605/10mg	60.5		
	Aloe emodin	Sigma- Aldrich	Analytical standard 311/10mg	31.1		

<i>Aloe vera</i> gel	Acemannan	Toronto Research chemicals	Technical grade-208.3/10mg	20.8	Chemical test for carbohydrates; Polysaccharide composition analysis by gas–liquid chromatography(8)	Molecular Exclusion Chromatography(90) UV-Vis Spectrophotometry(91)
	D-Mannose 6 phosphate	Sigma-Aldrich	≥98% (HPLC) (sodium salt)-354.0/100mg	3.5		None found
<i>Albizia coriaria</i> stembark	Lupeol	Sigma-Aldrich	Analytical standard-130/10mg	13.0	No monographs for <i>Albizia coriaria</i> stembark materials	HPLC-DAD quantification method for triterpenoids <i>Albizia lebbeck</i> (92)
	lupenone	Toronto Research chemicals	Analytical standard-170/2.5mg	68.0		HPLC-DAD quantification method for triterpenoids in <i>Albizia inundata</i> (93)
	Betulinic acid	Sigma-Aldrich/Supelco	Analytical standard 95.1/10mg	9.5		HPLC-DAD quantification method for betulinic acid in <i>Albizia lebbeck</i> (94)
			≥98% (HPLC)-70/5mg	14.0		
<i>Mangifera indica</i> leaf or stembark	Catechin	Sigma-Aldrich/Supelco	Analytical standard -307/10mg	30.7	No monographs for <i>Mangifera indica</i>	HPLC-DAD quantification method <i>Albizia lebbeck</i> (95),
	Mangiferin	Sigma-Aldrich/Supelco	Analytical standard-108/10mg	10.8		HPLC-UV quantification

					leaf or stembark materials	method for mangiferin (96)
	Catechin	Sigma-Aldrich/Supelco	Analytical standard - 307/10mg	30.7		HPLC-UV quantification method for phenolic compounds(97)
	Epicatechin	Not available	N/A			
	Gallic acid	Sigma-Aldrich	Phyproof® Reference Substance - 345/100mg	34.5		
	Quercetin	Sigma-Aldrich	United States Pharmacopeia (USP) Reference Standard- 357/200mg	1.8		
			phyproof® Reference Substance- 253/20mg	12.7		
			≥95% (HPLC)- 68.3/10g	6.8		
<i>Azadirachta indica</i> stembark or leaf	Azadirachtin	Sigma-Aldrich	Phyproof® Reference Substance- 403/5mg	80.6	WHO monographs- high-performance liquid chromatography quantification of tetranortriterpenes in oil and leaf materials (9)	HPLC-UV quantification method for Azadirachtins (98)
			~95%-249/0.5mg	498.0		
	Nimbin	Toronto Research chemicals	Analytical standard-105/1mg	105.0		HPLC-UV quantification method for nimbin(99)
	Nimbidin	Not available	N/A			None found
	Nimbolide	Sigma-Aldrich	≥98%-624/5mg	124.8		HPLC-UV quantification

	Mahmoodin	Not available	N/A			method for nimbolide(100) None found
<i>Zingiber officinale</i> rhizome	8-gingerol	Sigma- Aldrich/Supe lco	Analytical standard 489.0/10mg	48.9	WHO monographs- Thin-layer chromatography fingerprinting to identify gingerols and shogaols; quantitative gas chromatography and high-performance liquid chromatography analyses of ginger oils for gingerols, Shogaols(8)	HPLC-MS quantification methods for gingerols and related compounds(101) (102, 103)
	10-gingerol	Sigma- Aldrich	Analytical standard- 546/10mg $\geq 98\%$ (HPLC)-276/5mg phyproof® Reference Substance- 472/10mg	54.6 55.2 47.2		
	12-gingerol	Not available	N/A			
	6-gingerol	Sigma- Aldrich	Analytical standard- 448 /10mg $\geq 98\%$ (HPLC)- 358 /5mg phyproof® Reference Substance- 472/10mg	44.8 71.6 47.2		
	6-shogaol	Sigma- Aldrich	Analytical standard- 538/10mg Phyproof® Reference Substance- 657/10mg	53.8 65.7		

	Zingerone	Sigma-Aldrich	Analytical standard-84.50/50mg	1.7		None found
Warburgia ugandensis stembark	Polygodial	Sigma-Aldrich	≥97% (HPLC)-216/10mg	21.6	No monograph for <i>Warburgia ugandensis</i> stembark	None found
	Bemadienolide	Not available	N/A			None found
	Muzigadial	Not available	N/A			None found
	Warbuganal	Not available	N/A			None found
	Warbugadione	Not available	N/A			None found
	Warbugadial	Not available	N/A			None found
	Warbugin	Not available	N/A			None found
	Ugandensolide	Not available	N/A			None found
	Ugandensidial	Not available	N/A			None found

Selection of markers

Most of the compounds score points at least 5 points out of 8 except those for *Warburgia ugandensis* which scored only one point.

The scores of the markers for each plant are summarized in table 4.

Table 4: Ranking of the biomarkers

Plant material and manufacturer therapeutic claim	Bio-active compound	Evidence of Biological activity of bioactive compound	Level of evidence of biological activity related to traditional use of the material	Relative Concentration in plant or extract	Mean Concentration above (5 ppm) %ppm	Mean concentration above 50ppm Yes (1)/no (0) E	Reference Standard available Yes (1)/no (0) F	Analytical methods available ? Yes (1)/no (0)	Herb MaRS score
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Score		Yes-1 No-0 (A)	1 symptom-1 2 symptoms-2 3 symptoms-3 (B)		Yes (1) No (0) (C)	Yes (1) No (0) (D)	Yes (1) No (0) (E)	Yes (1) No (0) (F)	/8 (A+B+C +D+E+F)	
<i>Eucalyptus globulus</i> leaf/oil	aromadendrine	1	2	10-30% of essential oil (23)	1	1	1	1	7	
	α-terpeniol	1	3	0.50% of essential oil (23)	1	1	1	1	8	
	globulol	1	1	10-11% of essential oil (23)	1	1	1	1	6	
	1,8-cineol	1	3	97.32% of essential oil (104)	1	1	1	1	8	
<i>Aloe barbadensis</i> whole leaf/gel	Aloin A and B	1	3	0.1-0.6% of leaf, 10-30% of latex (105, 106)	1	1	1	1	8	
	Aloe emodin	1	2	0.09- 0.29 mg/g of whole leaf (107)	1	1	1	1	7	
	Acemannan	1	2	109-135ppm of gel (90)	1	1	1	1	8	
	Mannose phosphate	6	1	1	ND	0	0	1	0	3
<i>Albizia coriaria</i> stembark	Lupeol	1	3	1-6mg/g of stembark	1	1	1	1	8	
	lupenone	1	1	19-200ppm of stembark (93)	1	1	1	1	6	

	Betulinic acid	1	3	1.2-10mg/g of stem bark (94)	1	1	1	1	8
	Catechin	1	3	0.2-12mg/g of stembark (95)	1	1	1	1	8
<i>Mangifera indica</i> leaves/stem bark	Mangiferin	1	3	5-20mg/g of leaves (96)	1	1	1	1	8
	Catechin	1	3	71.4mg/g of stembark (97)	1	1	1	1	8
	Epicatechin	1	1	8.07mg/g of stembark (97)	1	1	0	0	4
	Gallic acid	1	3	2.08mg/g of stembark (97)	1	1	1	1	8
	Quercetin	1	3	0.76 to 1.16 mg/g of leaves (108)	1	1	1	1	8
<i>Azadirachita indica</i> leaves/stem bark/seeds	Azadirachtin A	1	3	3.8 to 4.8mg/g of seeds (109) 0.1-1mg/g of leaves (110)	1	1	1	1	8
	Nimbin,	1	3	0.018 to 0.64mg/g of oil (99)	1	1	1	1	8
	Nimbolide	1	2	0.9-6.7mg/g of leaf (100)	1	1	1	1	7
	Mahmoodin	1	2	Not determined	1	1	0	0	5
	12-gingerol	1	2	0.01-0.02mg/g of	1	1	0	1	6

<i>Zingiber officinalis</i> rhizome				rhizome (103)					
	10-gingerol	1	2	0.2-0.4mg/g of rhizome (101)	1	1	1	1	7
	8-gingerol	1	1	0.4-0.5mg/g of rhizome (101)	1	1	1	1	6
	6-gingerol	1	3	1.1-2.0mg/g of rhizome (101)	1	1	1	1	8
	6-shogaol	1	3	0.01-0.02mg/g of rhizome(103)	1	1	1	1	8
	Zingerone	1	2	ND	0	0	1	1	5
<i>Warbugia ugandensis</i> stembark	Bemadienolid e	1	0	ND	0	0	0	0	1
	Muzigadial,	1	1	ND	0	0	0	0	2
	polygodial	1	2	ND	0	0	1	0	3
	Warbuganal,	1	0	ND	0	0	0	0	1
	warbugadione	1	0	ND	0	0	0	0	1
	,								
	warbugadial,	1	0	ND	0	0	0	0	1
	warbugin	1	0	ND	0	0	0	0	1
	Ugandensidia l,	1	0	ND	0	0	0	0	1
	Ugandensolid e	1	0	ND	0	0	0	0	1

Key: ND=Not determined

Discussion

Phytochemical standardization is a key step in the quality assurance of herbal medicines and materials; it is essential to ensure reproducibility of the biological activity and quality of the product. In this study we established a list of compounds that can be used as markers for seven of the most commonly used plant materials in Uganda. Our emphasis was on compounds that are known to be active such that their determination informs both on quality and efficacy of the materials.

Markers for *Eucalyptus globulus* oil

Eucalyptus globulus oil is obtained from the leaves of *Eucalyptus globulus*. The oil used to manufacture products used to manage symptoms of respiratory tract disorders (cough, common cold, catarrh, sore throat, congestion from asthma, bronchitis, allergic conditions- sinusitis, rhinitis, hiccups, fever, measles symptoms), as pain balms and as mouth washes for mouth disorders (toothache, bad odor, sensitive teeth, bleeding gums, tooth cavities, tooth decay, and mouth sores) (6). Several compounds exhibit the pharmacological activities that support indications: Among the most studied are aromadendrine, α -terpineol, globulol, and 1-8, cineol (Figure 4); with scores of 7, 8, 6, and 8 respectively. Since all the compounds are available in pure form and several quantitative methods have been established, these compounds are all suitable as markers. The WHO and the African pharmacopoeia recommend the use of 1,8-cineol as a standard for both chemical reaction and TLC fingerprint identification methods (8, 11); however, some studies have shown aromadendrine to be major component, perhaps a multi-marker approach is more appropriate (23). These markers can be used to authenticate, determine phytoequivalence and monitor the consistence in quality of oils obtained from different subspecies and geographical locations.

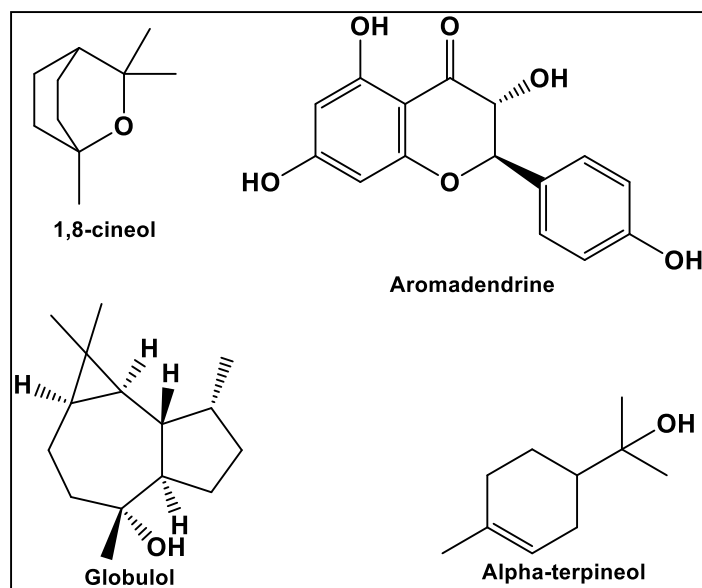


Figure 4: Bioactive compounds in *Eucalyptus globulus* oil

Markers for *Aloe vera* (*Aloe barberdensis*)

Aloe vera is used as the gel, latex or the whole leaf extract. The whole leaf and latex products are used to treat constipation and to “cleanse” the GIT, and to treat wounds (6). The main active ingredients are anthraquinone glycosides notably Aloin (barbaloin A and B) and aloe emodin(31) (Figure 5). The efficacy of these compounds is well established, their analytical standards are available and many analytical methods have been published: Therefore, aloin A and B scored 8 points while aloe emodin scored 7. Because of suspected carcinogenicity (111), some regulatory authorities have banned the inclusion of aloe (whole leaf and latex) in oral over the counter pharmaceuticals such as nutraceuticals and laxative products. For instance, the International Aloe Science Council set a limit of 10ppm total anthraquinone glycoside concentration (as aloin) while the European Medicines agency and Food and Drug authority set the limit at 0 ppm (112, 113). In this case aloin and aloe emodin are negative markers and can be used to assess the quality of OCT

products. However, the Uganda National Drug Authority and Uganda Bureau of standards have not published any regulations on the use of aloes.

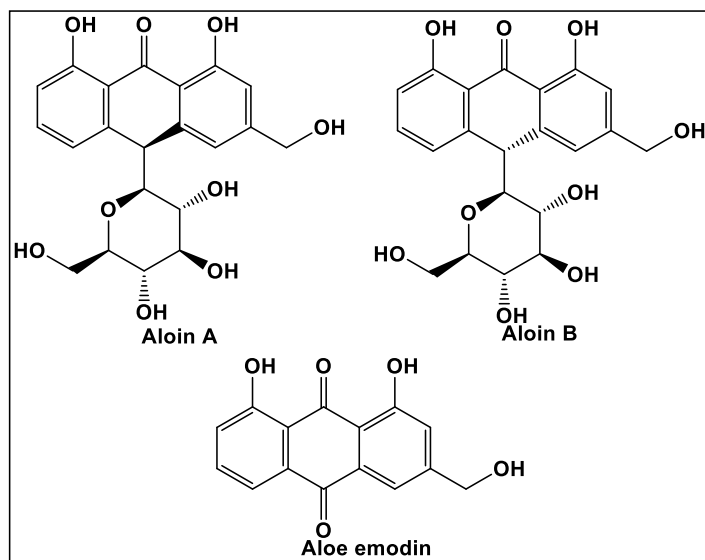


Figure 5: Bioactive compounds in *Aloe vera* whole leaf and latex

The main components of the gel are carbohydrates such as glucomannans and sugars (90) (Figure 6). One of the main compounds, acemannan scored 8 points. Its biological activity is well studied, analytical markers are available and analytical methods have been developed. The main sugar, mannose 6 phosphate scored only 3 points since its bioactivity is not well studied, there are no analytical methods, and thus its concentration in the gel has not been reported. For quality assurance of *Aloe vera* gel, the WHO monograph recommends a chemical test for carbohydrates and polysaccharide analysis by GC/MS; a molecular exclusion chromatographic method and a UV-Vis spectrophotometric method for polysaccharides have been published and are more affordable. TLC fingerprinting, chemical reaction tests for identification and spectrophotometric quantification of glycosides as aloin are recommended for the whole leaf and latex products (8).

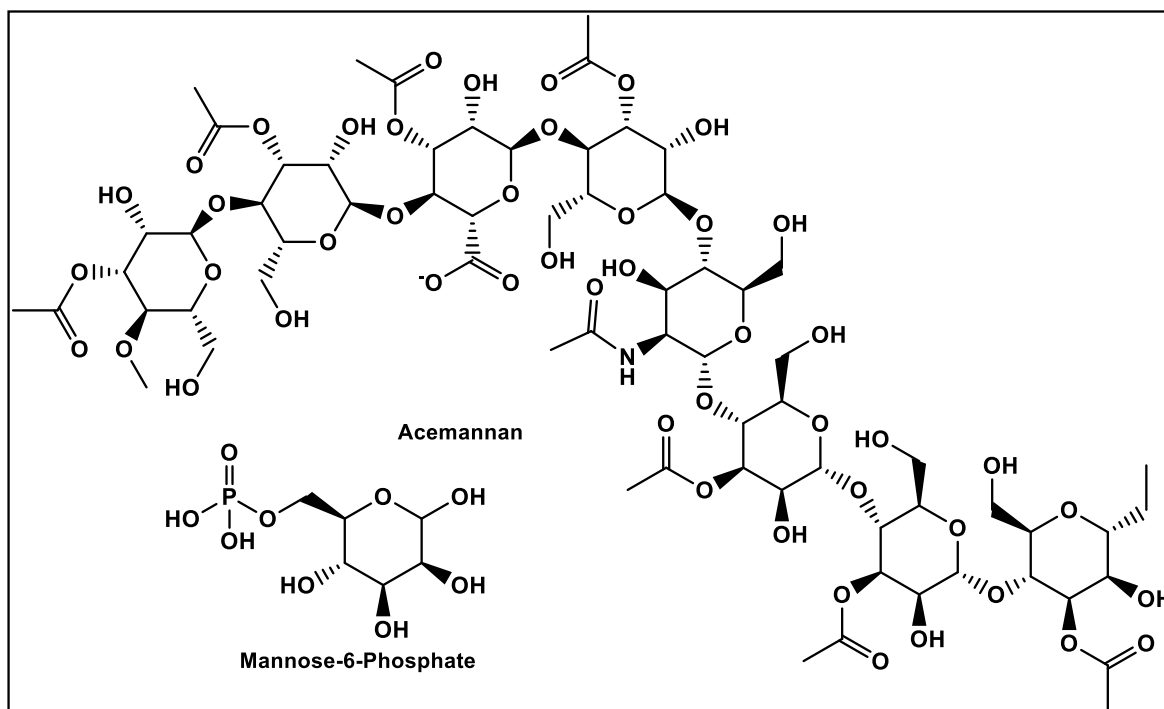


Figure 6: Bioactive compounds in aloe vera gel; Source for Acemannan structure: PubChem (114)

Markers for *Albizia coriaria*

The dried stem bark or is used as the drug; the commercial products are used for managing symptoms of respiratory tract disorders (whooping cough, catarrh, sore throat, congestion from asthma bronchitis, fever, sinusitis)(6). A few pharmacologically active compounds have been elucidated mainly the triterpenoids lupeol, lupenone, betulinic acid and betulin (115, 116) (Figure 7), with scores 8, 6, 8, 8 respectively. These compounds possess the biological activities relevant to the commercial uses of the products, are available in pure form, and have been quantified in many *Albizia* species although assay methods specific to *A. coriaria* are scarce. There are no pharmacopoeial methods or monographs for *A. coriaria* materials. The markers identified can be used to monitor the batch to batch consistency of raw materials since they sourced from the wild(117, 118) and to evaluated efficiency of extraction methods.

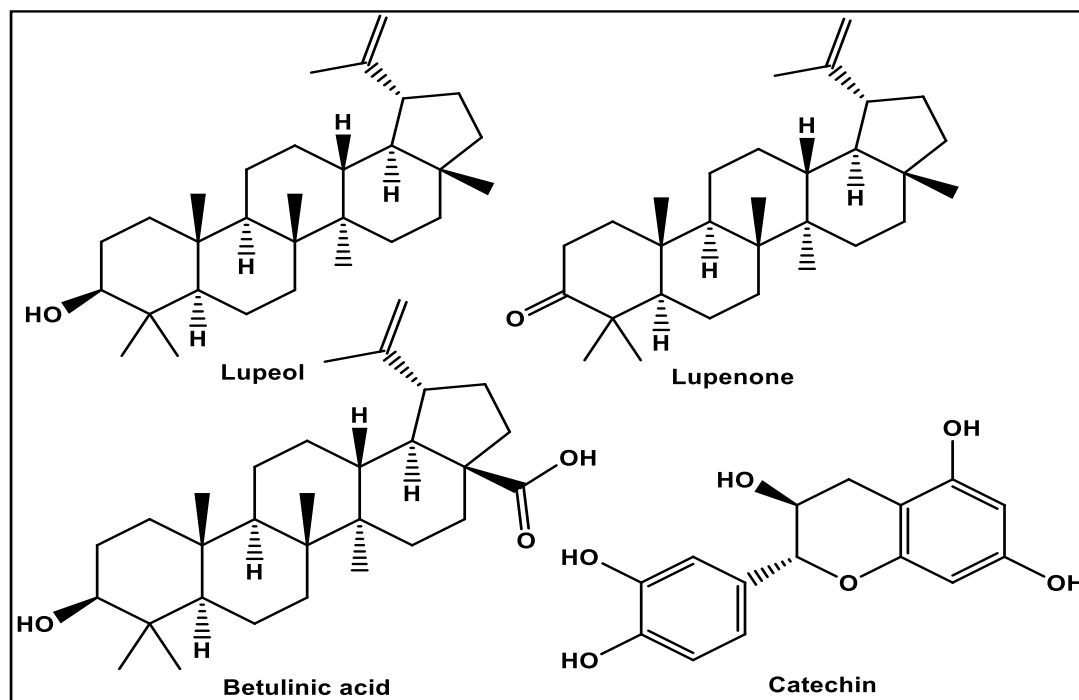


Figure 7: Bioactive compounds in *Albizia coriaria* stembark

Markers for *Mangifera indica*

The drug is obtained from extracts of the stembark, and leaves. The products containing *Mangifera indica* are used for management of respiratory tract disorders (whooping cough, catarrh, sore throat, congestion from asthma and bronchitis) (6). Several phenolic compounds (Figure 8) have been characterized and shown to poses biological activity relevant to the industrial use of the materials. Mangiferin, catechin and, quercetin and gallic acid scored 8, while epicatechin scored only 4 because it has limited evidence of biological activity, its analytical standard is not readily available and no analytical methods have been published. There are no monographs for *M. indica* materials. The markers identified can be used to monitor the batch to batch consistency of raw materials, to select the most suitable plant cultivars to source from (97), and to control the extraction and processing methods.

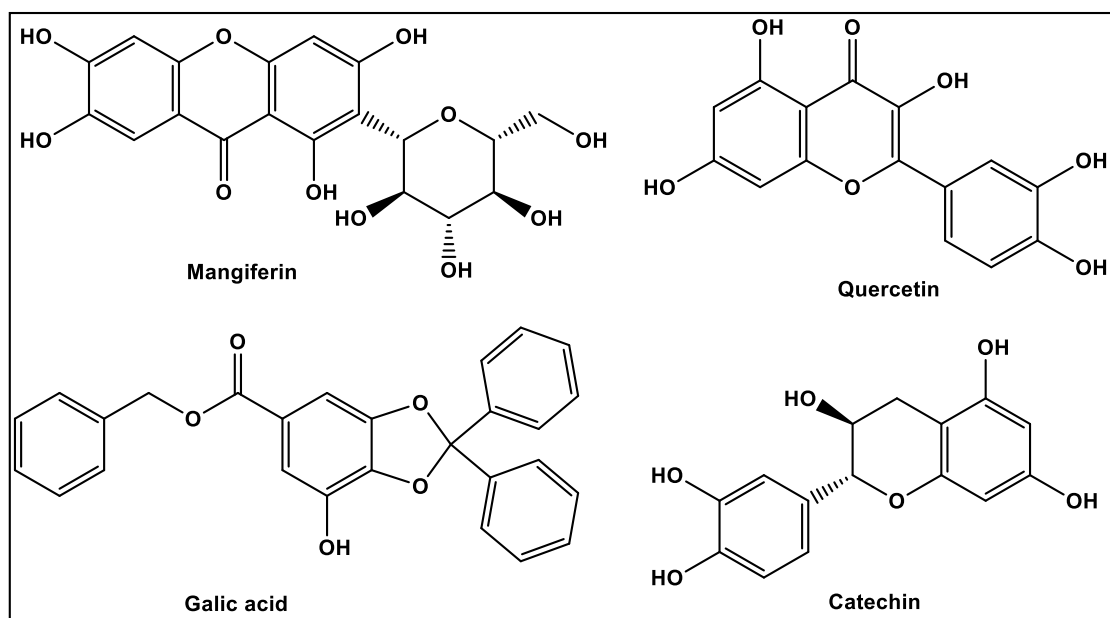


Figure 8: Bioactive compounds in *Mangifera indica*

Markers for *Azadirachta indica*

The seed oil, leaves and stem bark are used as the drug: the products are used to manage respiratory tract disorders (Cough, flu, sore throat, sinusitis), gastrointestinal disorders (gastric ulcers, flatulence, constipation) and as lip balms (dry, cracked, and painful lips) (6). The most important compounds are the tetranortriterpenes (72) including azadirachtin, nimbin, nimbidin, nimbolide and mahmoodin (Figure 9). All compounds scored 7 and above except nimbolide and mahmoodin which scored 5 points because of lack of analytical standards and assay methods.

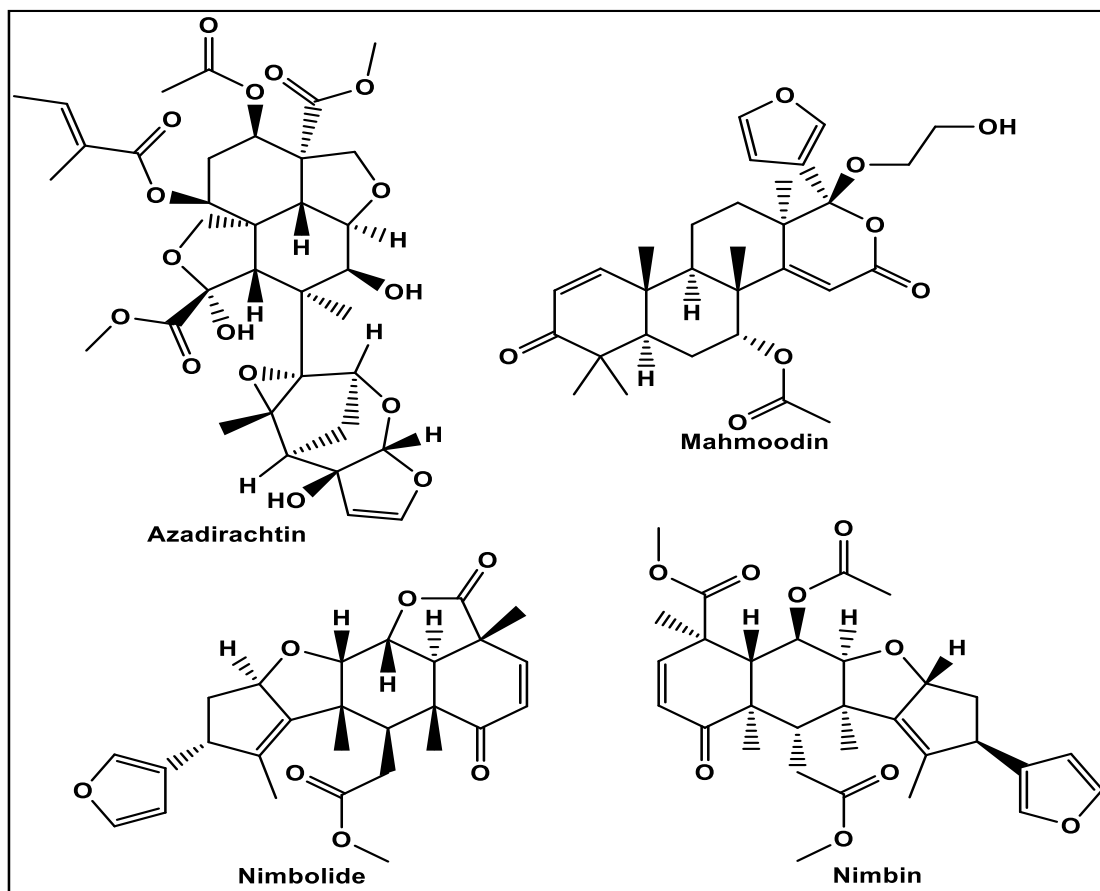


Figure 9: Bioactive compounds in *Azadirachta indica*

Markers for *Zingiber officinalis* (Ginger)

The drug is obtained from the extracts and used to manage symptoms of respiratory tract disorders (cough, flu, sore throat, sinusitis, bronchial asthma, and fever) (6). Gingerols and their dehydration products, the shogaols (Figure 10) have been extensively studied (77). The compounds possess several pharmacological activities relevant to the application of the products; their analytical standards are readily available (apart from 12-gingerol) and analytical methods have been published. Thus, all compounds scored between 6 and 8 points and are thus suitable as markers. The WHO monographs recommend TLC fingerprinting with gingerols and shogaols as standards; and GC and HPLC assay methods(8). The ratio of gingerols to shogaols can be used to determine

the freshness of the ginger samples, optimize extraction processes and to study phytoequivalence of gingers obtained from different sources.

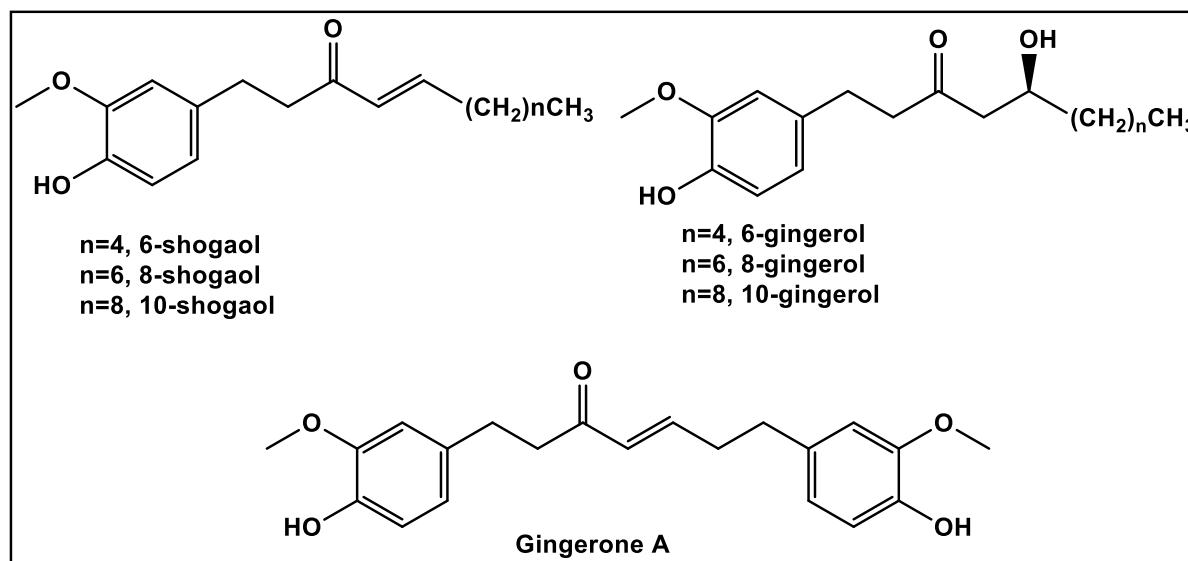


Figure 10: Bioactive compounds in *Zingiber officinalis*

Markers for *Warburgia ugandensis*

The bark of the stem is used as the drug for the treatment of respiratory tract disorders (cough, measles symptoms, common colds, sinusitis, rhinitis, asthma, catarrh, bronchial congestion, and hiccups) and gastric ulcers (6). While many compounds have been elucidated, the most important being the drimane sesquiterpenes (87) (Figure 11), specific bioactivity studies are rare. Only polygodial, muzigadial and muzigadiolide have been shown to possess some antimycobacterial activity (87). Besides limited pharmacological evidence, most of these compounds are not available in pure form for use as analytical standards and no assay methods have been published and there is no monograph for *Warburgia ugandensis* materials. Thus, the compounds scored between only 1 and 3 points.

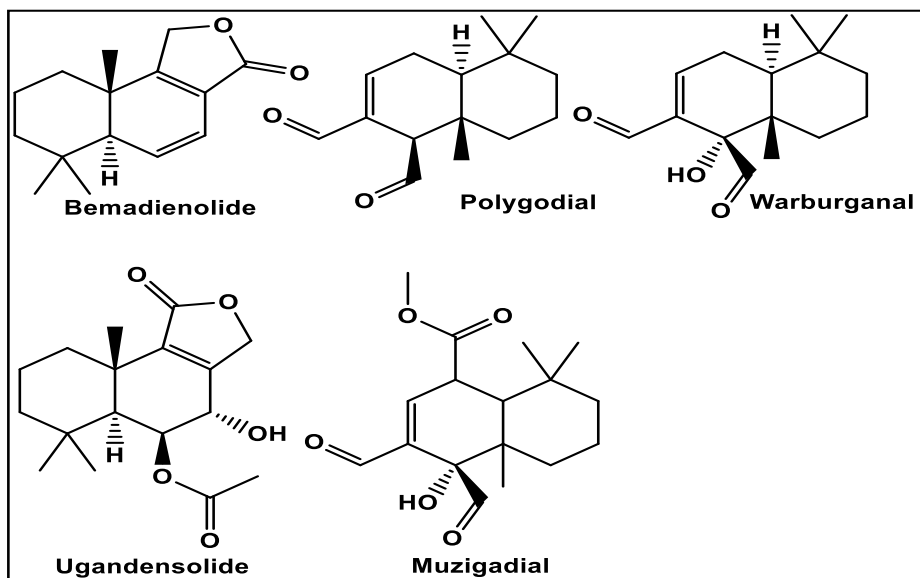


Figure 11: Bioactive compounds in *Warburgia ugandensis*

Conclusion

Markers have been identified for phytochemical standardization of seven most common medicinal plants in Uganda: the next step is to evaluate their suitability at the various stages of the production chain of the herbal medicines i.e., from authentication and quality control of raw materials to evaluating reproducibility in efficacy safety, and stability of finished products. Since most analytical standards are not specific, the use of multiple markers is recommended. The challenge with this approach is that analytical standards are expensive and some are not readily available. Besides quantification of marker compounds, construction of fingerprint databases for the various plants is encouraged. The standardized fingerprints can then be used for routine quality assessment of the plant materials.

DECLARATIONS

Ethical Approval

This work was approved by the Mbarara University of Science and Technology, Research and Ethics Committee.

Consent for Publication

Not applicable

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

Competing Interest

The authors declare that they have no competing interests

Funding

This study was funded by World Bank through Pharm-Biotechnology and Traditional Medicine Centre (PHARMBIOTRAC), African Center of Excellence II (ACE-II) Project. The funder contributed research money and stipends (SD) but has not involved in planning and implementation of the study.

Authors' Contribution

B.K conceived the idea, and drafted the manuscript; GA, E.I.M and H.K collected and analyzed bioactivity data; H.O and W.K collected and analyzed data on availability of analytical standards and assay methods; R.W draw the chemical structures; F.P.K and P.E.O supervised the work. All authors reviewed and approved the final manuscripts.

Acknowledgements

We appreciate the guidance and mentorship of Engineer Anke Weisheit (Business and Innovation manager), Dr. Casim Umba Tolo (Center Leader) of the Pharm-Biotechnology and Traditional Medicine Centre (PHARMBIOTRAC), Mbarara University of Science and Technology.

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