Sokoine University of Agriculture

PhD Thesis

Analytical Methods for Screening and Determination of Conventional Drugs Adulterated in Herbal Products

Christopher Johnson Mwankuna

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Analytical Methods for Screening and Determination of Conventional Drugs Adulterated in Herbal Products

Thesis submitted to Sokoine University of Agriculture in Fulfilment of the Requirements for Doctor of Philosophy

By

Christopher Johnson Mwankuna

Principal supervisor
Prof. Faith Philemon Mabiki

Co–supervisors
Dr. Eliapenda Elisante Mariki
Prof. Hamisi Masanja Malebo
Prof. Bjarne Styrishave

Department of Chemistry and Physics
College of Natural and Applied Sciences
Sokoine University of Agriculture, Morogoro, Tanzania

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Extended Abstract

Herbal products are popular worldwide. Their popularity is threatened by untrustworthy manufacturers who add conventional drugs. The addition of conventional drugs increases the risk of developing antimicrobial resistance and herb-drug interactions. To safeguard the users and enhance the safety of herbal products, analytical methods for screening and determining conventional drugs adulterated in herbal products are required. Therefore, this study was carried out to develop analytical methods and apply them in screening and determination of antibiotic, antimalarial, pain killer and erectile dysfunction adulterants in herbal products.

Thin layer chromatography methods for screening twelve conventional drugs in herbal products were developed and applied. The analytes were extracted from herbal products using a solvent mixture of acetonitrile:methanol:acetic acid:water (4:4:1:1, v/v). The mobile phase consisting of dichloromethane:ethyl acetate:methanol (75:15:10, v/v) separated well trimethoprim, sildenafil, paracetamol and sulfamethoxazole. Pyrimethamine, metronidazole and sulfadoxine were well separated by dichloromethane:ethyl acetate:methanol (77.5:12.5:10, v/v). In addition, acetyl salicylic acid, ibuprofen, diclofenac, quinine and lumefantrine were well separated by ethyl acetate:methanol:30% ammonia (75:22.5:2.5, v/v). Chromatographic separations were highly reproducible and more than 10 samples were analysed in one run. The developed methods were used to screen 229 herbal products. Consequently, 24.0% of the samples contained one adulterant while 21.4% contained at least two adulterants.

A high performance liquid chromatography–tandem mass spectrometry method was developed and used for screening and determining six conventional antibiotics (amoxicillin, ampicillin, metronidazole, trimethoprim, sulfamethoxazole,

and ciprofloxacin) in herbal products. The developed method had linear ($r^2 \geq 0.996$) calibration curves over the range of 0.005–2.5 µg mL^{-1} for all compounds except metronidazole, whose range was 0.005–1 µg mL^{-1}. The limit of detection ranged from 0.012 to 0.046 µg mL^{-1} while the limit of quantification ranged from 0.066 to 0.153 µg mL^{-1}. Accuracy, expressed as recovery of spiked herbal products ranged from 45% to 114%. The precision expressed as relative standard deviation at two concentration levels ranged from 1.6% to 15.9%. The matrix effect, expressed as matrix factor ranged from 0.79 to 0.92. The developed method was used to analyse 78 herbal products purchased from Njombe, Morogoro, Manyara, Arusha, Mwanza and Dar es Salaam in Tanzania. Metronidazole was detected in eight samples with the highest concentration of 1.38 µg g^{-1}.

Another high performance liquid chromatography–tandem mass spectrometry method was developed and used to screen and determine eleven conventional antimalarials (chloroquine, quinine, sulfadoxine, pyrimethamine, mefloquine, lumefantrine, amodiaquine, artemisinin, dihydroartemisinin, artesunate and artemether) in herbal products. The developed method had linear ($r^2 \geq 0.991$) calibration curves over the range of 0.001–0.3 µg mL^{-1} for all compounds. The limit of detection ranged from 0.002 to 0.02 μg mL^{-1} while the limit of quantification ranged from 0.006 to 0.08 μg mL^{-1}. Accuracy, expressed as recovery of spiked herbal products ranged from 52% to 128%. The precision, expressed as percent relative standard deviation at two concentration levels, ranged from 1.0% to 13.8%. The matrix effect, expressed as the matrix factor ranged from 0.77 to 0.97. The developed method was used to analyse 50 herbal product samples from Njombe, Morogoro, Manyara, Arusha, Mwanza and Dar es Salaam in Tanzania. Ten of the herbal products were found to contain amodiaquine, sulfadoxine, pyrimethamine, mefloquine, dihydroartemisinin, artemether and lumefantrine.

The developed thin layer chromatography and high performance liquid chromatography–tandem mass spectrometry methods are considered valuable tools for a better understanding of the adulteration of herbal products by addition of conventional drugs. The thin layer chromatography methods can be used for preliminary screening of herbal products prior to confirmation by other techniques such as high performance liquid chromatography–tandem mass spectrometry. On the other hand, confirmation and quantification of the selected antibiotic and antimalarial adulterants in herbal products can be achieved using the developed high performance liquid chromatography–tandem mass spectrometry methods.

Ikisiri Kuu

Dawa zitokanazo na mimea ni maarufu duniani kote. Umaarufu wake unahatarishwa na wazalishaji wasioaminika ambao huongeza dawa za kisasa na hivyo kusababisha hatari ya kutokea kwa usugu wa vimelea kwa dawa na mwingiliano wa dawa za kisasa na dawa zitokanazo na mimea. Mbinu za kisasa za uchambuzi zinahitajika ili kubaini na kutathmini dawa za kisasa zilizochanganywa na dawa zitokanazo na mimea na hivyo kulinda watumiaji na kuimarisha usalama wa dawa hizo. Kwa hiyo, utafiti huu ulifanyika ili kutengeneza mbinu za uchambuzi na kuzitumia katika kubaini dawa za kisasa zilizochanganywa na dawa zitokanazo na mimea.

Mbinu za *Thin layer chromatography* za kubaini dawa kumi na mbili za kisasa katika dawa zitokanazo na mimea zilitengenezwa na kutumika. Sampuli za dawa zitokanazo na mimea ziliandaliwa kwa ajili ya uchambuzi kwa kutumia mchanganyiko wa *acetonitrile:methanol:acetic acid:water* (4:4:1:1, *v/v*). Mchanganyiko wa *dichloromethane:ethyl acetate:methanol* (75:15:10, *v/v*) ulitenganisha vema *trimethoprim, sildenafil, paracetamol na sulfamethoxazole.* Mchanganyiko wa *dichloromethane:ethyl acetate:methanol* (77.5:12.5:10, *v/v*) ulitenganisha vema *pyrimethamine, metronidazole na sulfadoxine.* Aidha, *acetyl salicylic acid, ibuprofen, diclofenac, quinine* na *lumefantrine* zilitenganishwa vyema na mchanganyiko wa *ethyl acetate:methanol:30% ammonia* (75:22.5:2.5, *v/v*). Matokeo ya utenganishaji yaliweza kujirudia vizuri na zaidi ya sampuli 10 zilichambuliwa kwa mara moja. Mbinu zilizotengenezwa zilitumika katika kuchambua dawa 229 zitokanazo na mimea. Asilimia ishirini na nne (24.0%) ya sampuli ziligundulika kuwa na dawa ya kisasa moja wakati 21.4% zilikuwa na angalau dawa za kisasa mbili.

Mbinu ya *high performance liquid chromatography–tandem mass spectrometry* ilitengenezwa na kutumika kubaini

vijiuadudu sita vya kisasa (*amoxicillin, ampicillin, metronidazole, trimethoprim, sulfamethoxazole,* na *ciprofloxacin*) katika dawa zitokanazo na mimea. Mbinu hiyo ilikuwa na taswira nyoofu ($r^2 \geq 0.996$) kati ya 0.005–2.5 μg mL^{-1} kwa dawa zote za kisasa isipokuwa *metronidazole*, ambapo ilikuwa kati ya 0.005–1 μg mL^{-1}. Kiwango cha chini cha kubaini kilikuwa kati ya 0.012 hadi 0.046 μg mL^{-1} wakati kiwango cha chini cha kubaini kiasi kilikuwa kati ya 0.066 hadi 0.153 μg mL^{-1}. Usahihi, ulioonyeshwa kama uhuishaji ulianzia 45% hadi 114%. Utoshelevu, uliodhihirishwa kama achano sanifu–wiano katika viwango viwili vya kiasi ulianzia 1.6% hadi 15.9%. Athari ya ziada ilikuwa kati ya 0.79 hadi 0.92. Mbinu iliyotengenezwa ilitumika kuchambua dawa 78 zitokanazo na mimea zilizonunuliwa Njombe, Morogoro, Manyara, Arusha, Mwanza na Dar es Salaam, nchini Tanzania. *Metronidazole* ilipatikana katika sampuli nane na kiasi cha juu zaidi kilikuwa 1.38 μg g^{-1}.

Mbinu nyingine ya *High performance liquid chromatography–tandem mass spectrometry* ilitengenezwa ili kubaini na kutathmini dawa kumi na moja za kisasa za malaria (*chloroquine, quinine, sulfadoxine, pyrimethamine, mefloquine, lumefantrine, amodiaquine, artemisinin, dihydroartemisinin, artesunate* na *artemether*) katika dawa zitokanazo na mimea. Mbinu hiyo ilikuwa na taswira nyoofu ($r^2 \geq 0.991$) kati ya 0.001–0.3 μg mL^{-1} kwa dawa zote. Kiwango cha chini cha kubaini kilikuwa kati ya 0.002 hadi 0.02 μg mL^{-1} huku kiwango cha chini cha kubaini kiasi kilikuwa kati ya 0.006 hadi 0.08 μg mL^{-1}. Usahihi, ulioonyeshwa kama uhuishaji ulianzia 52% hadi 128%. Utoshelevu, uliodhihirishwa kama achano sanifu–wiano katika viwango viwili vya kiasi ulianzia 1.0% hadi 13.8%. Athari ya ziada ilianzia 0.77 hadi 0.97. Mbinu iliyotengenezwa ilitumika kuchambua sampuli 50 za dawa zitokanazo na mimea zilizonunuliwa Njombe, Morogoro, Manyara, Arusha, Mwanza na Dar es Salaam nchini Tanzania. Dawa kumi za mimea dawa zilipatikana kuwa na

amodiaquine, sulfadoxine, pyrimethamine, mefloquine, dihydroartemisinin, artemether na *lumefantrine*.

Mbinu zilizotengenezwa ni zana muhimu za kuleta taarifa sahihi za uchakachuaji wa dawa zitokanazo na mimea kwa kuongeza dawa za kisasa. Mbinu za *Thin layer chromatography* zilizotengenezwa zinaweza kutumika kwa uchunguzi wa awali wa dawa zitokanazo na mimea dawa kabla ya kuthibitishwa na mbinu zingine. Kwa upande mwingine, utambuzi na ukadiriaji wa vijiuadudu na dawa za malaria katika dawa zitokanazo na mimea vinaweza kufanyika kwa kutumia mbinu zilizotengenezwa za *high performance liquid chromatography–tandem mass spectrometry*.

Declaration

I, Christopher Johnson Mwankuna do hereby declare to the senate of Sokoine University of Agriculture that this thesis is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

…………………………… …………………………

Signature Date

Approved by;

Principal supervisor

………………………………… ……………………

Prof. Faith Philemon Mabiki Date

Co–supervisors

………………………………… ……………………

Dr. Eliapenda Elisante Mariki Date

A Malebo 18.05.2023
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Prof. Hamisi Masanja Malebo Date

Bjarne 17/5-2023
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Prof. Bjarne Styrishave Date

List of published papers

1. Mwankuna, C. J., Mariki, E. E., Mabiki, F. P., Malebo, H. M., Styrishave, B.& Mdegela, R.H. (**2023). ** Thin Layer Chromatographic Method for Detection of Conventional Drug Adulterants in Herbal Products. *Separations*, *10*, 23. https://doi.org/10.3390/separations10010023

2. Mwankuna, C. J., Uwamaliya, G. A., Mariki, E. E., Mabiki, F. P., Malebo, H. M., Mdegela, R.H. & Styrishave, B. (**2022). ** A HPLC–MS/MS method for screening of selected antibiotic adulterants in herbal drugs. *Anal. Methods*, 14, 1060–1068. https://doi.org/10.1039/d1ay01966j

3. Mwankuna, C. J., Kiros, F., Mariki, E. E., Mabiki, F. P., Malebo, H. M., Mdegela, R. H., & Styrishave, B. (**2023**). Optimization of HPLC–MS/MS method for determination of antimalarial adulterants in herbal products. Analytical Sciences, 39(3), 407–416. https://doi.org/10.1007/s44211–022–00255–8

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Words cannot express my gratitude to my parents, Johnson Ambilike Mwasakyeni (late) and Edah James Mwamajuja (late) for their awesome efforts and sacrifices of educating me. I also appreciate the prayers, care, support and love of my wife, Riziki, our children; Loveness, Ronel, Raziel, Reuel and Ramiel; and my siblings; Lutengano, Michael, Isaya, Loveness and Elisha (late). I would have not achieved this without their encouragement and support.

May God bless all those who supported and encouraged me throughout my programme of study.

Dedication

This study is dedicated to my late father and mother (Johnson and Edah) and my siblings; Lutengano., Michael, Isaya, Loveness and Elisha (late). I further dedicate to my lovely wife, Riziki and our children; Loveness, Ronel, Raziel, Reuel and Ramiel.

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List of symbols, abbreviations and acronyms

AMR	Antimicrobial Resistance
CE	Capillary Electrophoresis
CE–MS	Capillary Electrophoresis–Mass Spectrometry
GC	Gas Chromatography
GC–MS	Gas Chromatography–Mass Spectrometry
HPLC	High Performance Liquid Chromatography
HPLC–MS/MS	High Performance Liquid Chromatography–Tandem Mass Spectrometry
HPTLC	High Performance Thin Layer Chromatography
IR	Infrared
LC	Liquid Chromatography
LC–MS	Liquid Chromatography–Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MF	Matrix Factor
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometer
NIR	Near Infrared
NMR	Nuclear Magnetic Resonance
NSAIDs	Non–Steroidal Anti–Inflammatory Drugs
TLC	Thin Layer Chromatography
USA	United States of America
USD	United States Dollar

UV	Ultraviolet
WHO	World Health Organisation
mL	Millilitre
Mg	Milligram
G	Gram
µg	Microgram
v/v	Volume–volume ratio
µL	Microlitres
˚C	Degree Celsius

Chapter 1

General Introduction

1 Background information

1.1. Introduction

Herbal products are increasingly used worldwide as drugs, nutraceuticals and cosmetics (Sarker, 2014). Some of the products have been included in the national health care systems of various countries (Kala, 2017; Kasilo & Trapsida, 2013). Industry demand for herbal raw materials has also increased due to emergence of new products like health foods, natural cosmetics, and hygiene products (Nirmal et al., 2013). The perception that these products are safe, harmless and do not cause detrimental side effects has influenced their use (Tangkiatkumjai et al., 2020). In addition, patients with chronic illnesses and diseases that cannot be cured with conventional drugs seek treatment by herbal products (Calahan et al., 2016). Furthermore, approximately 88% of the World Health Organization (WHO) member states have formally developed policies, laws, regulations and programmes related to herbal products (WHO, 2019).

The wide use and increasing demand for herbal products has made it a lucrative product. The annual growth rate for world market of herbal products is estimated to be between 5% and 15% (Gromek et al., 2015; Nirmal et al., 2013). The global market of herbal products was USD 60 billion in 2017 and is estimated to increase to USD 104.78 billion by 2026 and to reach USD 5 trillion by 2050 (Bareetseng, 2022; Dhiman et al., 2016; Nirmal et al., 2013). The annual expenditures on herbal products in China, Korea and USA was USD 83.1 billion in 2012, USD 7.4 billion in 2009 and USD 14.8 billion in 2008, respectively (WHO, 2013). It is reported that non–powdered medicines valued USD 344,882 are traded in informal herbal products markets in

Tanzania per year (Mpelangwa et al., 2022; Posthouwer, 2015). The size of the market for herbal products has not been extensively assessed for many developing countries including Tanzania.

Herbal products have been reported to be adulterated with conventional drugs to enhance the claims stated on the label (Al Lawati et al., 2017; Ching et al., 2018; Nili-Ahmadabadi et al., 2019). Adulteration of herbal products is defined as "the intentional or unintentional addition of impurities or inferior products to a product or the removal of valuable portions from such products (Miller et al., 2001). Adulteration of herbal products with conventional drugs has been reported in some countries in Asia, Europe, Africa and America such as Malaysia, Taiwan, Singapore, Indonesia and China (Ariffin et al., 2021; Calahan et al., 2016; Fatmarahmi et al., 2022; Foroughi et al., 2017; Park et al., 2016; Pratiwi et al., 2021; Xu et al., 2019). Nevertheless, the impacts of such adulterants on the quality of herbal products remain poorly understood.

The use of herbal products adulterated with conventional drugs might increase the risk of developing antimicrobial resistance. In addition, the concurrent use of herbal products and conventional drugs can cause herb-drug interactions that may lead to serious clinical consequences. Hence, reliable quality control measures of herbal products may help to ensure good public health.

Adulteration of herbal products is a growing menace in Sub-Saharan African countries (Abuga, 2021; Dumba et al., 2021; Ofori-Kwakye et al., 2014). In Tanzania, traditional practitioners report that, some of their fellows add conventional drugs to their herbal preparations to convince the users that herbal preparations are potent (Otieno et al., 2014). However, the extent of such practices have not been established.

Often, multiple adulterants have been detected in a single product (Ching et al., 2018; Ren et al., 2012). In some cases the content of adulterants in herbal products has been found to be higher than the normal prescribed dosage of conventional drugs (Saberi et al., 2018). Most common adulterants are anorectic drugs, antidiabetic drugs, erectile dysfunction drugs and all kinds of pain killers as shown in Table 1 (Calahan et al., 2016; Ching et al., 2018; Saberi et al., 2018; Xu et al., 2019). Conventional drug adulterants may be approved drugs, banned drugs or drug analogues (Ching et al., 2018). For example erectile dysfunction drugs could be adulterated with sildenafil, tadalafil, vardenafil, as well as their analogues (Kee et al., 2018; Patel et al., 2014).

Table 1: Common conventional drug adulterants in herbal products

Categories of adulterants	Common adulterants
Non–steroidal anti–inflammatory drugs (NSAIDs) and Analgesics	Diclofenac, mefenamic acid, indomethacin, paracetamol, phenacetin, Ibuprofen and phenylbutazone
Anorectic	Sibutramine, rosiglitazone and metformin
Antibiotics	Amoxicillin
Corticosteroids	Dexamethasone, hydrocortisone, prednisolone, betamethasone, cortisone and triamcinolone
Antidiabetic agents	Chlorpropamide, tolbutamide and glibenclamide
Erectile dysfunction drugs	Sildenafil, tadalafil and vardenafil

Antimicrobial resistance (AMR) is the failure of antimicrobials' effect against the microorganisms (Mdegela et al., 2021; WHO, 2014). Improper use of antimicrobials and use of low quality antimicrobials are among the factors for emergence of AMR (Ayukekbong et al., 2017; Pokharel et al., 2019). Conventional drugs adulterated in herbal product at sub-therapeutic concentrations may render reduced potency and increases the chances of pathogens acquiring resistance (Ayukekbong et al., 2017; Bekoe et al., 2020; Kelesidis & Falagas, 2015). If strong and effective action against AMR is not taken, reports have estimated that about 10 million people will die across the world (Fig. 1) and Asia and Africa are projected to have high mortality rates attributable to AMR by 2050 (Table 2) (Neill, 2014; Pokharel et al., 2019). Therefore, combating AMR requires collective action including laboratory screening of herbal product for the presence of conventional drugs.

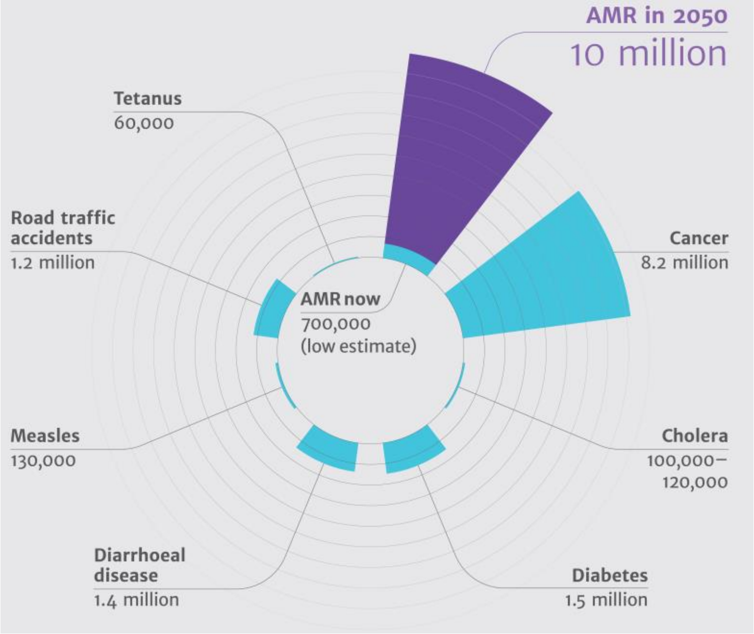

Figure 1: Comparison of deaths attributed to antimicrobial resistance to other causes every year

Table 2: Mortality rates by 2050 due to antimicrobial resistance in different regions

Regions	Mortality Rates by 2050
Asia	4,730,000
Africa	4,150,000
Europe	390,000
Latin America	392,000
North America	317,000
Oceania	22,000

Source: Dadgostar (2019)

The development of analytical methods to identify conventional drugs in herbal products is of great relevance. Analytical techniques that have been used for identification and quantification of adulterants in herbal products include thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and capillary electrophoresis (CE) (Moreira et al., 2014; Saeed et al., 2015). Due to herbal products' complex nature, generally, such techniques cannot be used for high confidence identification (Yu et al., 2016). Hyphenated techniques such as gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS) and capillary electrophoresis–mass spectrometry (CE–MS) improve the analytical selectivity and sensitivity and have been used for confirmation purposes.

TLC and HPTLC methods are simple and cheap methods that have been used to identify adulterants of in herbal products. Although many samples can be analysed simultaneously the methods are not particularly useful for confirmation of illegal adulteration due to low selectivity and suffers from low sensitivity (Pratiwi et al., 2021). Their selectivity and sensitivity can be improved by selecting an appropriate detection such as Raman spectroscopy and densitometric analysis (Minh et al., 2019). The methods are chosen when many samples are to be compared, when flexibility is important, and when rapid quantitative and semi-quantitative data are needed at low cost per sample. HPLC with UV detector offers advantages of higher sensitivity, separation power and reproducibility but is inadequate for high confidence identification. GC–MS is powerful technique for the analysis of herbal products but its applicability is determined by the volatility and thermal stability of analytes (Pratiwi et al., 2021). LC–MS whose selectivity and sensitivity are high is one of the most popular choices of technique for analysing complex herbal products matrices. The use of high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) in multiple reaction monitoring (MRM) mode is reported to offer excellent sensitivity and selectivity for the quantification of target analytes in herbal products. LC-MS/MS is capable of providing data on both the quantities and structures of compounds and is one of the most common techniques among other hyphenated tools that have been used for detection of adulterants in herbal products.

Rapid, reliable and effective method to screen out adulterated herbal products might be one of the most important strategies to deal with adulteration. For effective screening and quantitation of adulterants in herbal products, simultaneous and high throughput methods are desired because often multiple adulterants are found in a single product. In this study, TLC and HPLC–MS/MS methods for screening and determining conventional drug adulterants in herbal products were developed and applied.

Development of TLC methods involved selection of suitable mobile phase among the potential candidates (dichloromethane, methanol, ethyl acetate, acetone and acetic acid). The solvents were combined in various ratios taking into consideration their polarities to optimize the mobile phase for separation of the selected conventional drugs. HPLC–MS/MS method development comprised three main stages as shown in Fig. 2: (a) MS/MS spectra were recorded and mass spectrometry settings were optimized to find specific fragment ions for each compound, (b) A fast liquid chromatography method suitable for MS/MS detection was developed to separate the analytes from matrix components, (c) A cost-effective and selective sample preparation technique was selected and optimized. The developed HPLC–MS/MS methods were validated in terms of recovery, linearity, limits of detection and quantification, precision, and accuracy following the International Council for Harmonization (ICH) (ICH, 2005).

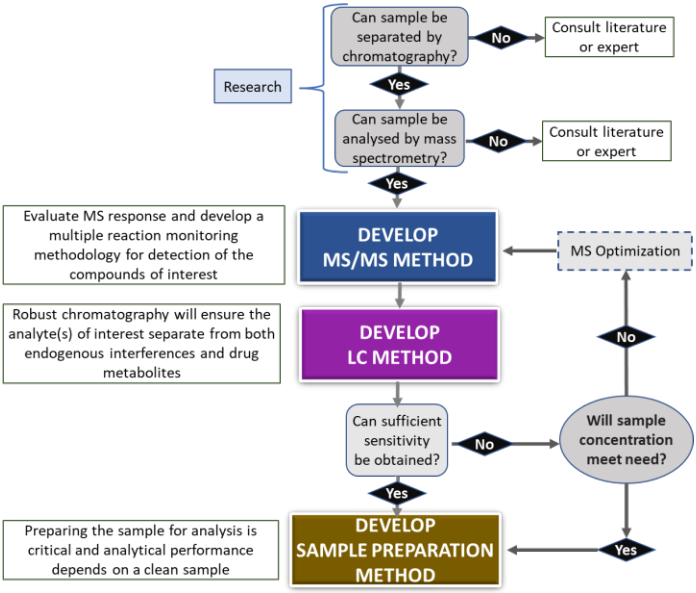

Figure 2: Workflow for HPLC–MS/MS method development

1.2. Statement of the problem and significance of the study

Despite the threat posed by adulteration in Tanzania, to the time this study was conceived, the information on extent of adulteration of conventional antibiotic, antimalarial, pain killer and erectile dysfunction drugs in herbal products was still limited. This was caused by the limited rapid and low cost laboratory analytical methods for screening herbal products. In this study, laboratory analytical methods were developed and can be used to detect and determine conventional drugs adulterated in herbal products. Screening of herbal product will ensure patients' safety, increased confidence, perpetuate global of herbal products and improve livelihood. This will further contribute to the curbing of antimicrobial resistance and promote the herbal products with bioactive compounds.

1.3. Research Objectives

1.3.1. Overall objective

To enhance the safety of herbal products through developing simple and rapid analytical techniques for detection and determination of conventional drug adulterants in herbal products.

1.3.2. Specific objectives

i. To develop thin layer chromatography (TLC) method for screening of herbal products for the presence of conventional drug adulterants.

ii. To develop HPLC–MS/MS methods for simultaneous determination of antibiotic adulterants in herbal products.

iii. To develop HPLC–MS/MS methods for simultaneous determination of antimalarial adulterants in herbal products.

1.4. Choice of conventional drugs, analytical methods and sample collection

1.4.1. Choice of conventional drugs

Selection of conventional drugs based on their common usage in Tanzania. Conventional drugs for treatment of malaria (quinine, sulfadoxine, pyrimethamine, artemether, chloroquine, piperaquine, mefloquine, lumefantrine, amodiaquine, artemisinin, dihydroartemisinin, artesunate and artemether), for bacterial infections (metronidazole, trimethoprim, sulfamethoxazole, ampicillin, amoxicillin and ciprofloxacin) for pain (diclofenac, acetyl salicylic acid and ibuprofen) and for erectile dysfunction (sildenafil) were selected for the study.

1.4.2. Choice of analytical methods

TLC methods were selected because they are simple, rapid, inexpensive, can use several different solvents and can analyse several samples at a time. HPLC–MS/MS methods were selected due to their high sensitivity and selectivity especially when operated in multiple reaction monitoring (MRM).

1.4.3. Collection of samples and analysis

Herbal product samples were collected from business cities of Dar es Salaam, Mwanza and Arusha as well as urban areas of Njombe, Morogoro and Manyara regions in Tanzania. With the assistance of traditional and alternative medicine coordinators in the Councils, traditional and alternative medicine service centres were identified. Herbal products described for various ailments were randomly obtained from shops, street vendors, open markets, clinics and home-based practitioners using covert method of sampling from July 2018 to April 2019.

Analyses of samples were carried out in the Chemistry Laboratory at the Department of Chemistry and Physics, Sokoine University of Agriculture, Tanzania and the

Toxicology Laboratory, Department of Pharmacy, University of Copenhagen, Denmark. A total of 229 samples were screened using the developed TLC methods (Appendix 1). The developed HPLC–MS/MS method for screening antibiotic adulterants was used to screen 78 herbal product samples Appendix 2. In addition, the developed HPLC–MS/MS method for determination of antimalarial adulterants was used to analyse 50 herbal product samples (Appendix 3).

1.5. Limitation of the study

The developed TLC methods are cost saving and easy to operate. However, they have poor sensitivity, specificity, and accuracy as compared with HPLC and HPLC–MS/MS. The developed HPLC–MS/MS methods are expensive and require trained personnel. The developed TLC methods can be used for preliminary screening of herbal products for the presence of adulterants. However, confirmation of the identified adulterants using other methods such as HPLC–MS/MS will be required.

1.6. Structure of the thesis

The thesis is organised in a published paper format. Chapter one entails the general introduction to this study. Chapters two through four presents published papers. Chapter five presents the general discussion of the study and chapter six presents the general conclusions and recommendations of the study.

Chapter 2

Thin Layer Chromatographic Method for Detection of Conventional Drug Adulterants in Herbal Products ‡

Christopher Johnson Mwankuna [1,*], Eliapenda Elisante Mariki [1], Faith Philemon Mabiki [1], Hamisi Masanja Malebo [2], Bjarne Styrishave [3] and Robinson Hammerton Mdegela [4]

[1] Department of Chemistry and Physics, College of Natural and Applied Sciences, Sokoine University of Agriculture, Morogoro P.O. Box 3038, Tanzania.
[2] UNESCO National Commission of the United Republic of Tanzania, P.O. Box 20384, Dar es Salaam, Tanzania.
[3] Toxicology Laboratory, Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark.
[4] Department of Veterinary Medicine and Public Health, College of Veterinary and Biomedical Sciences, Sokoine University of Agriculture, P.O. Box 3021, Morogoro Tanzania.

* Correspondence: cmwankuna@sua.ac.tz

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separations

Article

Thin Layer Chromatographic Method for Detection of Conventional Drug Adulterants in Herbal Products

Christopher Johnson Mwankuna [1,*], Eliapenda Elisante Mariki [1], Faith Philemon Mabiki [1], Hamisi Masanja Malebo [2], Bjarne Styrishave [3] and Robinson Hammerton Mdegela [4]

[1] Department of Chemistry and Physics, College of Natural and Applied Sciences, Sokoine University of Agriculture, Morogoro P.O. Box 3038, Tanzania
[2] UNESCO National Commission of the United Republic of Tanzania, Dar es Salaam P.O. Box 20384, Tanzania
[3] Toxicology Laboratory, Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark Universitetsparken 2, DK-2100 Copenhagen, Denmark
[4] Department of Veterinary Medicine and Public Health, College of Veterinary and Biomedical Sciences, Sokoine University of Agriculture, Morogoro P.O. Box 3021, Tanzania
* Correspondence: cmwankuna@sua.ac.tz

Abstract: Commercially available conventional drugs have been used to adulterate herbal products. Considering the rapid growth of herbal products' market, it is essential to screen herbal products for the presence of conventional drugs. Simple analytical methods are needed for the rapid screening of conventional drugs that are likely to be adulterated in herbal products. Thin layer chromatography (TLC) methods for screening twelve conventional drugs in herbal products have been developed and applied. The analytes were extracted from herbal products using acetonitrile:methanol:acetic acid:water (4:4:1:1, v/v). Solvent mixture of dichloromethane:ethyl acetate:methanol (75:15:10, v/v) separated well trimethoprim, sildenafil, paracetamol, and sulfamethoxazole while pyrimethamine, metronidazole, and sulfadoxine were well separated by dichloromethane:ethyl acetate:methanol (77.5:12.5:10, v/v). In addition, acetyl salicylic acid, ibuprofen, diclofenac, quinine, and lumefantrine were well separated by ethyl acetate:methanol:30% ammonia (75:22.5:2.5, v/v). Chromatographic separations were found to be highly reproducible, and more than 10 samples can be analysed in one run. The method was applied in the screening of 229 herbal products. Consequently, 24.0% of the samples contained one adulterant, while 21.4% contained at least two adulterants. All conventional drugs detected in herbal products were not mentioned on the labels and therefore the consumers are kept unaware of their side effects and health problems. Further studies for confirming and quantitatively determining the adulterants in a wide range of products as well as a systematic toxicological analysis of the adulterants in herbal products are recommended.

Keywords: adulteration; herbal drugs; synthetic drugs; herbal medicines

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1. Introduction

The use and demand for herbal products in both developed and developing countries is increasing [1]. The adulteration of herbal products is a growing menace in sub-Saharan African countries [2–4]. Adulteration in the context of herbal products is the intentional addition of undeclared drugs/chemical substances or substitution with non-drug components, or the addition of foreign non-drug materials into a product [5,6]. In Tanzania, traditional health practitioners have reported that some of their colleagues add conventional drugs to their herbal preparations to compel the users that herbal preparations are potent [7]. Cheating on the efficacy of some herbal products has been reported where adulteration with conventional drugs is performed by some practitioners to position the herbal product brand in the market [8]. The adulteration of herbal products with conventional drugs has been reported in Asia, Europe, Africa, USA, and Brazil [4,9–11]. Nevertheless, the extent and levels of the adulteration of herbal products remain poorly documented.

The adulteration of herbal products with conventional drugs pose health risks. The use of adulterated herbal products may result into herbal–drug interactions that can lead to adverse health problems [12–15]. Moreover, the use of herbal products containing variable amounts of conventional drugs may contribute to antimicrobial resistance. However, the impacts of such adulterants on the quality of herbal products remain poorly understood.

The ever-increasing popularity of herbal-based medications dictates that the national and international regulatory bodies assess the quality of herbal products. The screening of herbal products for the presence of conventional drugs is an integral part in assessing the quality of herbal products. However, the methods/techniques for screening the quality of herbal products are limited. Consequently, most herbal products remain unscreened for adulteration. This calls for the development of an easy and rapid method/technique for screening the quality of herbal products in the market. Different methods are reported for the detection of conventional drugs in herbal products. Capillary electrophoresis has been used for the analysis of adulteration in herbal medicines and dietary supplements for weight control [16]. Thin layer chromatography (TLC), high-performance thin layer chromatography (HPTLC), and high-performance liquid chromatographic (HPLC) have been used for the determination of erectile dysfunction drugs and steroids in herbal preparations [17,18]. High-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) has been used for the screening and determination of erectile dysfunction drugs and antibiotics in herbal products [19–21]. Thin layer chromatography (TLC) is simple, less expensive, and easy to execute, and its components have low electrical power requirements [22]. For instance, the separation step does not require power, and, when indicator chemicals are used for visualization, it can be applied using a hand-operated sprayer. However, when an ultraviolet (UV) lamp, which is a common visualization light system, is used, power is required. TLC is also fast and can analyse several samples at a time and, with appropriate modifications, can be taken to the field [23–27]. Other chromatographic techniques such as gas chromatography (GC) and high-performance liquid chromatographic (HPLC) can be used for the detection of conventional drugs, although the costs for their continuous operation are higher than those for TLC. Mass spectrometric (MS) detection is a most convenient tool for confirmation; however, it is not available in many laboratories, or the laboratory infrastructure does not make their continuous use possible. TLC can be used for screening the conventional drugs in laboratories where there is an irregular supply of electricity or a limited budget for using GC and HPLC techniques and where the application of mass spectrometric detection is not feasible. Therefore, TLC is widely used for the screening of samples before using confirmatory techniques and in resource-limited settings. In addition, the simultaneous analysis of samples and standards statistically increases its analytical precision and accuracy [28]. In this study, simple and rapid TLC methods that can be used for screening purposes prior to more demanding techniques were developed and used to detect conventional drugs mentioned in Table 1. The selection of conventional drugs was based on their common usage in Tanzania. During the optimization of the mobile phase's composition, an emphasis was placed on achieving the best possible overall separation of the selected conventional drugs.

Table 1. Structures of the selected conventional drugs for the developed method.

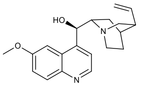
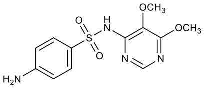
Compound Name	Chemical Structure
Quinine	
Sulfadoxine	

Table 1. *Cont.*

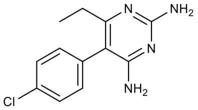
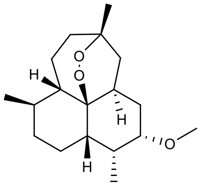
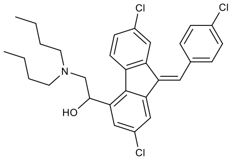
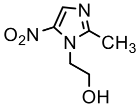
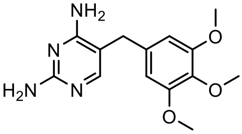
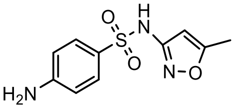
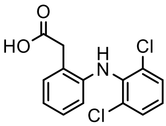
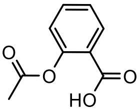
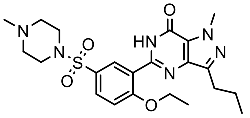
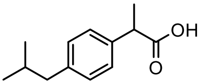
Compound Name	Chemical Structure
Pyrimethamine	
Artemether	
Lumefantrine	
Metronidazole	
Trimethoprim	
Sulfamethoxazole	
Diclofenac	
Acetyl salicylic acid	
Sildenafil	

Table 1. *Cont.*

Compound Name	Chemical Structure
Ibuprofen	

2. Materials and Methods

2.1. Materials, Reagents and Chemicals

Reagents used included HPLC grade methanol (Finar, India, 99.8%), HPLC grade acetonitrile (Finar, India, 99.9%), water for HPLC (Carlo Erba, France), HPLC grade ethyl acetate (Loba Chemie, India, 99.5%), dichloromethane (Finar, India, 99.9%), and analytical grade 30% ammonia solution (Loba Chemie, India). Precoated TLC sheets (ALUGRAM Xtra SIL G/UV$_{254}$, Macherey-Nagel, Germany) were used as the chromatographic plates. Chromatographic developments were carried out in a rectangular glass and chromatographic spots were visualized using ultraviolet lamps emitting 254 and 366 nm radiation. Analytical standards of metronidazole, trimethoprim, sulfamethoxazole, sildenafil, paracetamol, pyrimethamine, sulfadoxine, acetyl salicylic acid, ibuprofen, diclofenac, quinine, and lumefantrine were from Sigma Aldrich. All analytical standards had purity above 99%.

2.2. Preparation of Standards and Samples

Individual reference standard solutions were prepared by dissolving each analytical standard in methanol, producing 1.0 mg mL^{-1}, and they were stored at −20 °C. Mixture of standards were prepared at 0.2 mg mL^{-1} using methanol as diluent.

Next, 5 g of the sample in a 100 mL Erlenmeyer flask 20 mL of the extraction solvent mixture (acetonitrile:methanol:acetic acid:water, 4:4:1:1, *v*/*v*) was added. The mixture was sonicated for 20 min. It was then passed through Whatman filter paper (No. 1) before analysis.

2.3. Optimization of Mobile Phase Composition for TLC Method

The optimal mobile phase compositions for the separation of the selected conventional drugs were achieved by testing solvents of various compositions of ethyl acetate and methanol; dichloromethane, ethyl acetate, and methanol; dichloromethane, ethyl acetate, and acetone. Individual standard solutions were spotted on the 20 cm × 10 cm TLC plates, which had been divided into 20 strips with width of 1.0 cm. Aliquots of 5 μL of each of the standards were spotted on TLC plates at a distance of 1 cm using a micropipette. The plates were developed in a closed glass chamber containing developing solvents having prior saturation at 25–30 °C. Chromatograms were observed on UV light at 254 nm and retention factors (R_f) were established for each analyte using Equation (1). In addition, the peak shape and the separation were visually assessed.

$$R_f = \frac{\text{Distance moved by the analyte}}{\text{Distance covered by the mobile phase}} \tag{1}$$

2.4. Application of the Optimized Method

2.4.1. Sample Collection

Herbal products described for various ailments were randomly obtained from shops, street vendors, open markets, clinics, and home-based practitioners using the covert method of sampling from July 2018 to April 2019. The address of the centres were gathered from district coordinators for traditional and alternative medicine. Large business cities of Dar es Salaam and Arusha as well as the urban areas of Njombe and Manyara regions in Tanzania were included in the study. Of the 229 collected samples, 34 were from Arusha, 45 were from Dar es Salaam, 64 were from Manyara, and 86 were from Njombe. Samples were bought from shops (30%), street vendors (29%), clinics (22%), home-based practitioners (17%), and open markets (2%). The major health problems indicated on the labels were bacterial infections (24%), painkiller (31.9%), malaria (17.5%), erectile dysfunction (10.9%), diabetes (8.3%), and others (7.4). Most of the samples were packed in well-designed plastic bottles (75.4%). Others were packed in paper bags (17.2%), used water bottles (5.3%), and some were wrapped in pieces of newspaper (2.1%). All samples were in powder form and, after purchase, were stored in a freezer to maintain their integrity.

2.4.2. Analysis of Collected Samples

Aliquots of 5 µL of each of the sample extracts were spotted on the TLC plates using a micropipette and two spots of a mixture of standards were also applied. The plates were developed in a closed glass chamber containing developing solvents. Similarities between the retention factors of detected spots from samples with standards led to the identity of analytes in the samples. Figure 1 shows a photograph of the chromatogram for the separation of the samples and a mixture of standards.

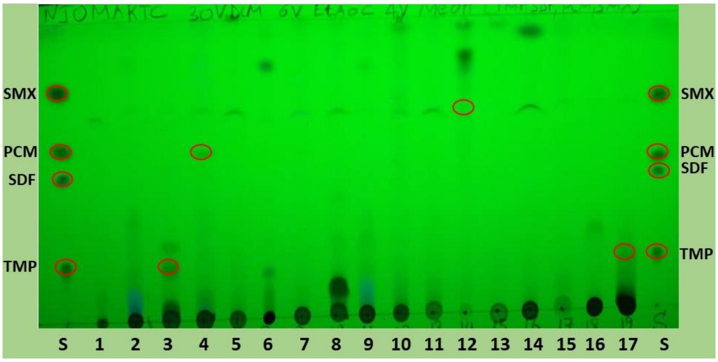


Figure 1. Chromatogram obtained at the separation of samples 1–17 and a mixture of standards, S containing, SMX—sulfamethoxazole, PCM—paracetamol, SDF—sildenafil, and TMP—trimethoprim using dichloromethane:ethyl acetate:methanol (75:15:10, *v*/*v*) and detected in UV light at 254 nm.

3. Results and Discussion

3.1. Optimization of Mobile Phase Composition for TLC Methods

Solvent development usually required 25–40 min and the pre-saturation of the TLC chamber with a mobile phase for at least 30 min, which led to a good separation with reproducible retention factor (R_f) values, as also observed in previous studies [29,30]. The attempt to separate all the twelve compounds (metronidazole, trimethoprim, sulfamethoxazole, sildenafil, paracetamol, pyrimethamine, sulfadoxine, acetyl salicylic acid, ibuprofen, diclofenac, quinine, and lumefantrine) on a single TLC plate was not successful, as several compounds had very close values of retention factor. This could be due to the similar retaining ability of the compounds rendered by the characteristics of the compounds and mobile phase [31]. Based on the tested solvents and their compositions, metronidazole, trimethoprim, sulfamethoxazole, sildenafil, paracetamol, pyrimethamine, and sulfadoxine were best separated by dichloromethane:ethyl acetate:methanol (75:15:10, *v*/*v*). The retention factors of sulfadoxine and sulfamethoxazole, as well as those of sildenafil and metronidazole, were very close, such that two different runs, with minor changes in composition (method I and II), were proposed for the best visualization, as indicated in Table 2. A slight increase in dichloromethane and a slight decrease in ethyl acetate lead to increased retention factors of metronidazole and sulfadoxine. On the other hand, acetyl salicylic acid, ibuprofen, diclofenac, quinine, and lumefantrine were best separated by ethyl acetate:methanol:30% ammonia (75:22.5:2.5, v/v).

Table 2. Solvent composition, separated compounds, and their retention factors for the developed methods.

Method	Solvent Composition	Separated Compounds	Retention Factor
I	Dichloromethane:ethyl acetate:methanol (75:15:10, v/v)	Trimethoprim Sildenafil Paracetamol Sulfamethoxazole	0.15 0.39 0.60 0.79
II	Dichloromethane:ethyl acetate:methanol (77.5:12.5:10, v/v)	Pyrimethamine Metronidazole Sulfadoxine	0.26 0.48 0.84
III	Ethyl acetate:methanol:30% ammonia (75:22.5:2.5, v/v)	Acetyl salicylic acid Ibuprofen Diclofenac Quinine Lumefantrine	0.16 0.26 0.35 0.60 0.91

3.2. Application of the Optimized Method

3.2.1. Characterization of Collected Herbal Samples

Most of the products' labels (66%) did not indicate the composition and amount of the different ingredients in the products, the manufacturer's address, dosage, manufacturing, and expiry dates. None of the labels indicated the presence of the conventional drugs in the product. About 86.3% of the labelled products were marked as being a 100% natural product. Very few samples (5.9%) indicated the composition of the product, while 9.7% described the storage conditions. Relatively large number of samples (58.2%) indicated the dosage of the herbal product. Therapeutic indications were presented on all labels, and, in most cases, it was indicated that a single product can treat at least five ailments. Similar observations have been reported elsewhere [32,33]. Products packed in paper bags, used water bottles, and pieces of newspaper may raise concerns about the hygiene under which products are prepared.

3.2.2. Adulteration of Herbal Products

Out of 229 screened samples, 24.0% contained one adulterant, while 21.4% contained at least two adulterants. Samples collected from the Njombe region presented the highest adulteration rates (51%), followed by Dar es Salaam (47%), Manyara (47%), and Arusha (26). The adulterants found in the screened products were trimethoprim, sulfamethoxazole, pyrimethamine, paracetamol, sulfadoxine, metronidazole, sildenafil, lumefantrine, acetyl salicylic acid, diclofenac, ibuprofen, and quinine. This is a likely result of the ill practice of some traditional health practitioners to capture the market for their products. Adding conventional drugs to herbal preparations by untrustworthy traditional health practitioners have been noted elsewhere [7]. As indicated in Table 3, half of the samples from herbal shops, street vendors, and open markets contained adulterants. Herbal clinics and home-based practitioners, respectively, had 39% and 37% of samples that contained adulterants. A high percentage of adulteration for the products from herbal shops and street vendors may be attributed to the dealers aspiring to position their herbal product brand in the market and hence make a large profit. Similarly, the adulteration of herbal products with varieties of conventional drugs has been observed in other counties [2,3,8,18].

3.2.3. Frequency of Adulterants

The most common adulterants are listed in order of the frequency of detection (with the incidents of detection totalling above four) in Table 4. Trimethoprim (43), sulfamethoxazole (28), pyrimethamine (27), paracetamol (25), sulfadoxine (19), and metronidazole (15) were among the most frequently encountered adulterants. Up to eight adulterants were detected in a single sample. Almost quarter of the screened products (24.0%) contained one adulterant, and 21.4% contained at least two adulterants. The presence of more than one adulterant in a product was observed by other researchers [9,34]. Trimethoprim and sulfamethoxazole occur in a combination termed as cotrimoxazole or commonly septrin. In Tanzania, cotrimoxazole is used as a prophylactic agent for HIV and AIDS patients and in diarrheal diseases for non-HIV patients [35,36]. Its high frequency of adulteration could be targeting the prevention of opportunistic infections in HIV/AIDS patients and to manage diarrhoea. A combination of pyrimethamine and sulfadoxine is recommended for intermittent preventive treatment of malaria during pregnancy [37]. The objective of its adulteration could to render clinical effectiveness for malaria treatment. The adulteration of paracetamol and metronidazole could be targeting pain and protozoa infections, respectively.

Table 3. Adulteration of herbal products according to the supplying sources.

Source	No. of Samples	No. of Adulterated Samples (%)
Herbal Clinics	51	20 (39.2)
Home-Based	40	15 (37.5)
Herbal Shops	68	34 (50.0)
Street Vendor	66	33 (50.0)
Open Market	4	2 (50.0)
Total	229	104

Table 4. Frequency of adulteration of herbal products.

Ranking of Adulteration	Detected Adulterant	Frequency of Adulteration
1	Trimethoprim	43
2	Sulfamethoxazole	28
3	Pyrimethamine	27
4	Paracetamol	25
5	Sulfadoxine	19
6	Metronidazole	15
7	Sildenafil	11
8	Lumefantrine	11
9	Acetyl salicylic acid	9
10	Diclofenac	8
11	Ibuprofen	7
12	Quinine	5

3.2.4. Distribution of Adulterants among Therapeutic Areas

Categorizing herbal products according to their claimed indications as shown in Table 5 reveal that half of the products claimed to be pain killers and almost half (47.3%) of the products claimed for bacterial infections contained adulterants. Pain and bacterial infections have been cited as most frequent health problems for which people use herbal products [38]. In addition, other studies reported that products purportedly for pain killing and fever were most commonly adulterated [39,40]. As also observed by Huang et al. [34], some adulterants appeared in categories not relevant to their claimed indication or purpose. For example, although antidiabetic conventional drugs were not screened in the products, antidiabetic products were found to contain other conventional drugs such as trimethoprim, sulfadoxine, and ibuprofen. While consumers are assuming that they are taking a natural product, they could unknowingly be exposed to dangerous complications such as unpredictable herb–drug interactions which could increase or decrease an undesirable pharmacological effect of either or both components.

Table 5. Adulterated herbal products according to therapeutic areas.

Indications	No. of Screened Samples	No. of Adulterated Samples	% Adulterated Samples
Antibacterial	55	26	47.3
Antidiabetic	19	8	42.1
Antimalarial	40	17	42.5
Pain killer	73	37	50.7
Erectile dysfunction	25	8	32.0
Others	17	8	47.1

4. Conclusions

Simple and rapid TLC methods for the detection of metronidazole, trimethoprim, sulfamethoxazole, sildenafil, paracetamol, pyrimethamine, sulfadoxine, acetyl salicylic acid, ibuprofen, diclofenac, quinine, and lumefantrine in herbal products were developed. Chromatographic separations were found to be highly reproducible, and more than 10 samples can be analysed in one run. The proposed TLC methods are simple, rapid, and flexible compared to HPLC, and they can be used for the preliminary screening of herbal products for the presence of adulterants. However, confirmation of the identified adulterants using other methods such as HPLC-MS/MS will be required. The presence of adulterants in herbal products might unknowingly be exposing consumers to conventional drugs that may lead to herb–drug interactions. The screening of the herbal products using the developed methods may contribute to the protection of herbal product consumers from unethical practices such as adulteration. We recommend further studies for the confirmation and quantitative determination of the adulterants in a wide range of products as well as a systematic toxicological analysis of the adulterants in herbal products.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to ethical considerations.

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Conflicts of Interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Chapter 3

A HPLC–MS/MS Method for Screening of Selected Antibiotic Adulterants in Herbal Drugs [‡]

Christopher Johnson Mwankuna, *[a] Grace Ange Uwamaliya, [b] Eliapenda Elisante Mariki, [a] Faith Mabiki, [a] Hamisi M. Malebo, [c] Robinson Mdegela [d] and Bjarne Styrishave [b]

[a] Department of Chemistry and Physics, College of Natural and Applied Sciences, Sokoine University of Agriculture, P. O. Box 3038, Morogoro, Tanzania.
[b] Toxicology Laboratory, Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark.
[c] UNESCO National Commission of the United Republic of Tanzania, Magogoni Street, P. O. Box 20384, Dar es Salaam, Tanzania
[d] Department of Veterinary Medicine and Public Health, College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, P. O. Box 3015, Morogoro, Tanzania

* Correspondence: cmwankuna@sua.ac.tz

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A HPLC-MS/MS method for screening of selected antibiotic adulterants in herbal drugs

Christopher Johnson Mwankuna, *[a] Grâce Ange Uwamaliya,*[b]
Eliapenda Elisante Mariki, [a] Faith Mabiki,[a] Hamisi M. Malebo,[c] Robinson Mdegela[d]
and Bjarne Styrishave[b]

The use of herbal products adulterated with conventional drugs increases the risk of developing microbial resistance and causes herb-to-drug interaction, leading to severe clinical consequences. The complex nature of herbal products has been a challenge for the unambiguous identification of adulterants. The improved analytical selectivity and sensitivity of hyphenated techniques such as high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) enable the confirmatory screening of adulterants in herbal products. Simultaneous screening of adulterants is necessary and efficient because it has been established that more than one chemical adulterant may be present in one herbal product. An HPLC-MS/MS method for the simultaneous detection and quantification of amoxicillin, ampicillin, metronidazole, ciprofloxacin, sulfamethoxazole, and trimethoprim in powdered herbal drugs was developed. Deuterated metronidazole-d3, trimethoprim-d3, ciprofloxacin-d8, and sulfamethoxazole-d4 were used as internal standards (ISs). For each analyte, two transitions were monitored using protonated molecules as precursor ions. The extraction of analytes from herbal products was performed using a simple methanol : water : formic acid (90 : 10 : 0.05, v/v) extraction solvent. Chromatographic separation was done in a gradient of 0.01% formic acid in methanol and 0.01% formic acid in MilliQ water. The calibration curves were linear ($r^2 \geq 0.996$) over the range of 0.005–2.5 μg mL^{-1} for all compounds except metronidazole, whose range was 0.005–1 μg mL^{-1}. The limit of detection (LOD) ranged from 0.012 to 0.046 μg mL^{-1}, while the limit of quantification (LOQ) ranged from 0.066 to 0.153 μg mL^{-1}. The accuracy, expressed as the recovery of spiked herbal products, ranged from 45% to 114%. The precision, expressed as relative standard deviation (RSD) at two concentration levels, ranged from 1.6% to 15.9%. The matrix effect expressed as the matrix factor (MF) ranged from 0.79 to 0.92. The developed method was applied to powder herbal products purchased in Tanzania. Amoxicillin, ampicillin, trimethoprim, sulfamethoxazole, and ciprofloxacin were not detected in all samples. Metronidazole was detected in eight samples with the highest concentration of 1.38 μg g^{-1}. The developed method is suitable for the detection of all the studied antibiotic adulterants in herbal products. Quantification can be performed for all the compounds except ciprofloxacin due to its lower recovery.

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1 Introduction

Herbal products are increasingly used worldwide as medicines, nutraceuticals, and cosmetics. Their use has been influenced by the broad spread perception that the products are safe and harmless and do not have detrimental side effects.[1] Also, patients with chronic illnesses and diseases that cannot be cured with conventional drugs seek treatment with herbal products.[1,2] Along with their increased use, herbal products are sometimes adulterated by the addition of conventional drugs to enhance the claims stated on the label.[3,4] The use of adulterated herbal products increases the risk of developing microbial resistance[5] and causes herb-to-drug interaction, leading to severe clinical consequences.[6–8] Hence, reliable quality control measures of herbal products may help to ensure good public health.

Analytical techniques that have been used for the identification and quantification of adulterants in herbal products include thin layer chromatography (TLC), high performance

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[a] *Department of Chemistry and Physics, College of Natural and Apllied Sciences, Sokoine University of Agriculture, P. O. Box 3038, Morogoro, Tanzania. E-mail: cmwankuna@sua.ac.tz*

[b] *Toxicology Laboratory, Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark Universitetsparken 2, DK-2100 Copenhagen, Denmark. E-mail: mkn752@alumni.ku.dk*

[c] *UNESCO National Commission of the United Republic of Tanzania, Magogoni Street, P. O. Box 20384, Dar es Salaam, Tanzania*

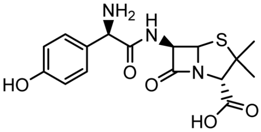
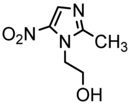
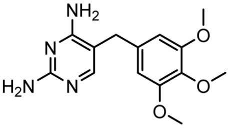
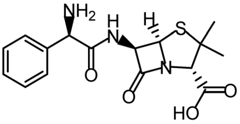
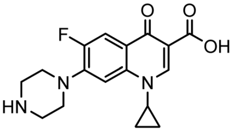
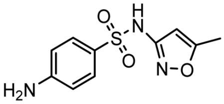
[d] *Department of Veterinary Medicine and Public Health, College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, P. O. Box 3015, Morogoro, Tanzania*

liquid chromatography (HPLC), and capillary electrophoresis (CE).[9–12] Due to the complex nature of herbal products, generally, such techniques cannot be used for high confidence identification.[13] Hyphenated techniques such as gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS) and capillary electrophoresis/mass spectrometry (CE/MS) greatly improve the analytical selectivity and sensitivity and have been used for confirmation purposes. The use of high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) in multiple reaction monitoring (MRM) mode is reported to offer excellent sensitivity and selectivity for the quantification of target analytes in herbal products.[14–16]

Methods for screening several potential adulterants simultaneously in a single run are necessary and efficient because it has been established that more than one chemical adulterant may be present in one herbal product.[16,17] Studies have reported methods for simultaneous determination of antibiotics in various matrices but not in herbal products.[18–20] Although the simultaneous determination of tetracycline antibiotic adulterants in herbal drugs has been reported, there are no such reports for other antibiotics.[14] In addition, the screening of adulterants in herbal products in Tanzania is not performed.

The purpose of this study was to develop simple and selective analytical methods for the detection and determination of antibiotic adulterants in herbal products using simple extraction procedures, HPLC separation, and tandem mass

Table 1 Optimized MS/MS parameters for the determination of the selected antibiotics

Compound	Chemical structure	Precursor ion $[M + H]^+$ (m/z)	Cone voltage (V)	Product ions[a] (m/z)	
				Quantification	Confirmation
Amoxicillin		366.13	10	349.0 (10)	207.9 (12)
Metronidazole		172.5	16	127.9 (12)	110.8 (20)
Trimethoprim		291.17	26	122.8 (24)	229.9 (22)
Ampicillin		350.14	12	105.8 (16)	173.9 (14)
Ciprofloxacin		332.21	22	288.1 (16)	231.0 (36)
Sulfamethoxazole		254.13	18	155.9 (14)	107.8 (22)

[a] Collision energy (in volts) used to obtain each product ion is shown in parentheses.

spectrometric detection. To the best of our knowledge, such an approach comprising antibiotic adulterants has not been reported so far. Amoxicillin, ampicillin, metronidazole, ciprofloxacin, sulfamethoxazole, and trimethoprim (structures are shown in Table 1), being the commonly used antibiotics in Tanzania, were selected as antibiotic adulterants for this study.

2 Materials and methods

2.1 Solvents and chemicals

HPLC grade methanol and formic acid were purchased from VWR Chemicals (BDH, USA). Ultrapure water was obtained using a Milli-Q® UF-Plus apparatus (Millipore Corp., Burlington, MA, USA). Analytical standards of amoxicillin, ampicillin, ciprofloxacin, metronidazole, trimethoprim, and sulfamethoxazole were purchased from Sigma Aldrich (Glostrup, Denmark). Deuterated trimethoprim-d3, ciprofloxacin-d8, sulfamethoxazole-d4, and metronidazole-d3 (d*n*, where *n* is the number of deuterium atoms in a molecule of the compound), used as internal standards, were purchased from Toronto Research Chemicals (TRC, Canada). All analytical standards had purity above 99%. Solaray fermented turmeric, a pure herbal product containing turmeric, was bought from a local herbal shop in Copenhagen, Denmark, and used for the spike recovery studies.

2.2 HPLC-MS/MS instrumentation and optimization

An Agilent 1100 liquid chromatography system (Agilent Technologies, Germany) coupled to a Quattro Micro API triple quadrupole Mass Spectrometer (Waters, Micromass Limited, UK) was used. The LC systems consisted of a Degasser, G1379A; Binary pump, G1312A; Autosampler G1313A; Column oven, G1316A; Diode array detector, G1315B. Separations were performed on a Kinetex analytical column: C18, (2.1 × 75 mm; 2.6 μm) and a 10 mm guard column (Phenomenex, USA). The chromatography system was coupled to a Quattro Micro API triple quadrupole Mass Spectrometer (Waters, Micromass Limited, UK) equipped with an electrospray ionization (ESI) interface. The acquisition and treatment of data were done using Masslynx 4.1 software (Waters Corporation, USA). The nebulizing and collision gases were nitrogen and argon, respectively.

The ion source parameters were optimized to obtain adequate and reproducible responses for the analytes and internal standards. To select the optimal MS/MS parameters for each analyte, individual standard solutions prepared in 0.1% formic acid in water : methanol (95 : 5, v/v) were first evaluated by infusing them into the MS. Both the positive and negative ion modes were tested. The molecular ions were selected for collision-induced dissociation to generate characteristic MS/MS spectra for each compound. Two specific precursor–product reaction transitions were selected for each compound. For each compound, the most intense and the next most intense daughter ions were selected for quantification and confirmation, respectively. The Multiple Reaction Monitoring (MRM) mode was employed for data acquisition.

Different mobile phase compositions and additives were tested to optimize chromatographic separation. For the aqueous phase, formate/formic acid buffer and MilliQ with formic acid (FA) and for the organic phase, formic acid in either methanol or acetonitrile were tested. Acidic mobile phases were chosen as they improve the ionization efficiency of compounds. Varying concentrations of methanol, acetonitrile, formic acid, and formate were investigated. Furthermore, the flow rate and column temperature were optimized to improve chromatographic resolution and peak shapes and reduce the total analysis time.

2.3 Preparation of standards

Individual stock solutions were prepared by dissolving each analytical standard in methanol, making 1000 μg mL^{-1}, and were stored at −20 °C. Individual working standards and mixtures were prepared by diluting the stock solutions with 0.01% formic acid in water : methanol (95 : 5, v/v). For MS optimization, each stock solution was diluted to 10 μg mL^{-1}. The calibration standards were prepared by diluting a mixture of standards at 100 μg mL^{-1} in methanol to 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0 and 10.0 μg mL^{-1}. Each calibration standard contained 0.5 μg mL^{-1} of the mixture of internal standards.

2.4 Sample preparation optimization

2.4.1 Absolute and relative recoveries. Sample preparation involved extraction, evaporation, and filtration. Extraction was performed to isolate the analytes from the sample. Evaporation was carried out in order to exchange the organic solvent used for extraction with the aqueous solvent for the best chromatographic separation. Filtration was performed to eliminate any particulates that would affect the column and HPLC performance. The absolute and relative recoveries were investigated for every sample preparation step, *i.e.* filtration, evaporation, and extraction, as well as for the entire procedure. Absolute recoveries of 80–120% and relative recoveries of 60–140% were acceptable. A pre-and post-spiking approach was used as described below; for each step, the final concentration of analytes was expected to be 1.0 μg mL^{-1}.

1. A mixture of antibiotic standards of known volume and concentration was spiked into four aliquots of a sample. The pre-spiked samples were studied through the step of interest, and finally, a mixture of internal standards was spiked before analysis.
2. A mixture of internal standards of known volume and concentration was spiked into four aliquots of a sample. The pre-spiked samples were carried out through the step of interest, and finally, a mixture of the antibiotic standards was spiked before analysis.
3. Four aliquots of a sample were prepared where no pre-spiking was conducted; instead, both the antibiotic standards and the internal standards were post-spiked.

Absolute and relative recovery for each analyte were calculated using eqn (1) and (2) respectively.

$$\text{Absolute recovery (\%)} = \frac{\text{area}_{\text{pre-spiked analyte}}/\text{area}_{\text{post-spiked IS}}}{\text{area}_{\text{post-spiked analyte}}/\text{area}_{\text{post-spiked IS}}} \times 100\% \quad (1)$$

$$\text{Relative recovery (\%)} = \frac{\text{absolute recovery of target analyte}}{\text{absolute recovery of IS}} \times 100 \quad (2)$$

2.4.1.1 *Recoveries for the filtration step.* Twenty μL of 100 μg mL^{-1} antibiotic standard mixture were made up to 1 mL with the mobile phase mixture A : B (95 : 5, v/v), and it was filtered through a 0.22 μm cellulose acetate syringe filter. The filter was washed with 1 mL of mobile phase mixture. The filtrates were combined and were post-spiked with 20 μL of 100 μg mL^{-1} internal standard mixture. The mixture was made up to 2 mL and taken for analysis.

Twenty μL of 100 μg mL^{-1} internal standard mixture were made up to 1 mL with the mobile phase mixture A : B (95 : 5, v/v) and it was filtered through a 0.22 μm cellulose acetate syringe filter. The filter was washed with 1 mL of mobile phase mixture. The filtrates were combined and were post-spiked with 20 μL of 100 μg mL^{-1} antibiotic standard mixture. The mixture was made up to 2 mL and taken for analysis.

One mL of mobile phase mixture A : B (95 : 5, v/v) was filtered through a 0.22 μm cellulose acetate syringe filter. The filter was washed with 1 mL of mobile phase mixture. The filtrates were combined and were post-spiked with both 20 μL of 100 μg mL^{-1} antibiotic standard mixture and 20 μL of 100 μg mL^{-1} internal standard mixture. The mixture was made up to 2 mL and taken for analysis.

2.4.1.2 *Recoveries for the evaporation step.* Twenty μL of 100 μg mL^{-1} antibiotic standard mixture were made up to 2 mL with methanol. 1 mL of MilliQ water was added, and the mixture was evaporated to 1 mL at 25 °C under a gentle flow of nitrogen gas. The residues were post-spiked with 20 μL of 100 μg mL^{-1} internal standard mixture and made up to 2 mL with the mobile phase mixture before analysis.

Twenty μL of 100 μg mL^{-1} internal standard mixture were made up to 2 mL with methanol. 1 mL of MilliQ water was added and the mixture was evaporated to 1 mL at 25 °C under a gentle flow of nitrogen gas. The residues were post-spiked with 20 μL of 100 μg mL^{-1} antibiotic standard mixture and made up to 2 mL with the mobile phase mixture before analysis.

A mixture of 2 mL methanol and 1 mL of MilliQ water was evaporated to 1 mL at 25 °C under a gentle flow of nitrogen gas. The residues were post-spiked with both 20 μL of 100 μg mL^{-1} antibiotic standard mixture and 20 μL of 100 μg mL^{-1} internal standard mixture and made up to 2 mL with the mobile phase mixture before analysis.

2.4.1.3 *Recoveries for the combined extraction, evaporation, and filtration steps.* A pure turmeric herbal product bought in Denmark was used to study the extraction recoveries. It was a fine, yellow powdered product. To 1 g of the sample, 100 μL of 100 μg mL^{-1} antibiotic standard mixture were spiked. It was thoroughly mixed and allowed to dry for one hour. It was extracted twice with 5 mL of methanol : water : formic acid (90 : 10 : 0.05, v/v) using a tilt shaker for 30 minutes. It was centrifuged (5300×*g*, 5 min) after each extraction. A mixture of 2 mL of the combined supernatant and 1 mL of MilliQ was evaporated to 1 mL at 25 °C under a gentle stream of nitrogen gas. The sample was transferred to a 2 mL Eppendorf tube, post-spiked with 20 μL of 100 μg mL^{-1} of the internal standard mixture, made up to 2 mL by adding the mobile phase and centrifuged (30 000×*g*; 5 min). The supernatant was collected for analysis.

2.5 Sample preparation

A 50 μL aliquot of the mixture of IS solution (1 μg mL^{-1} in methanol) was added to 1.0 g of sample in a clean 12 mL glass centrifuge tube. It was thoroughly mixed and allowed to dry for one hour. After adding 5 mL of methanol : water : formic acid (90 : 10 : 0.05, v/v), the sample was vortex-mixed for 30 seconds, followed by tilt shaking for 30 minutes. The sample was then centrifuged (5300×*g*; 5 min). The supernatant was transferred into a 12 mL vial. The extraction step was repeated, and the extracts were combined and vortex-mixed for 30 seconds. A mixture of 2 mL of the combined extract and 1 mL of Milli-Q water was evaporated to 1 mL at 25 °C under a gentle stream of nitrogen gas. The sample was transferred to a 2 mL Eppendorf tube, made up to 2 mL by adding the mobile phase and centrifuged (30 000×*g*; 5 min). The supernatant was transferred to an autosampler vial, and a volume of 5 μL was injected into the chromatography system.

2.6 Method validation

For the validation of the analytical procedure, the International Council for Harmonization (ICH) was followed. The method performance was evaluated by verification of the linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), selectivity and matrix effect.

Calibration curves were constructed using standards at nine concentration levels. The curves were best fitted using a least-squares linear regression model $y = mx + b$, in which y is the peak area ratio of analyte to IS, m is the slope of the calibration curve, b is the y-axis intercept of the calibration curve and x is the analyte concentration. The accuracy (expressed as recovery) was studied by a pre- and post-spiked approach described as described in Sections 2.4.1.3. The precision was investigated using standards at 1.0 and 0.01 μg mL^{-1} each with eight injections. The instrument limit of detection (LOD) was determined from the calibration curves as three times the ratio of residual standard deviation of the linear regression divided by the slope. Furthermore, the limit of quantification (LOQ) was determined as ten times the same ratio. No interfering peaks were observed at the retention times of the analytes and IS. The matrix effect expressed as the matrix factor (MF), which is described by eqn (3),[21] was evaluated by comparing the mean area response of post-extraction spiked samples with the mean area of neat standards at 1 μg mL^{-1}. MF > 1, signifies ion enhancement, MF < 1 signifies ion suppression and MF = 1 implies that the analytical method is free from the matrix effect. Practically, matrix factors below 0.85 (ion suppression) or above 1.15 (ion enhancement) would imply that a matrix effect was present.[22] In addition, the use of deuterated internal standards, as used in this study, corrects the matrix effect.[23]

$$\text{Matrix factor (MF)} = \frac{P_{\text{post-spiked}}}{P_{\text{neat solution}}} \tag{3}$$

where $P_{\text{post-spiked}}$ is the average analyte peak response of an extracted blank matrix sample spiked with the analyte after extraction and $P_{neat\ solution}$ is the average analyte peak response of a reference solution at the same nominal concentration.

3 Results and discussion

3.1 HPLC-MS/MS optimization

The optimal source parameters were: capillary 3.2 kV, exit potential 3 V, RF lens 0.2 V, source temperature 120 °C, desolvation temperature 450 °C, and desolvation and cone gas flow rates 650 and 100 L min^{-1} respectively. The precursor ions of all the compounds and Internal Standards (ISs) were protonated molecular ions $[M + H]^+$. The analytical parameters were optimized for each compound, as shown in Table 1. The dwell time was set at 100 ms for each channel. The observed precursor–product transitions were also reported in previous studies.[18–20,24–26]

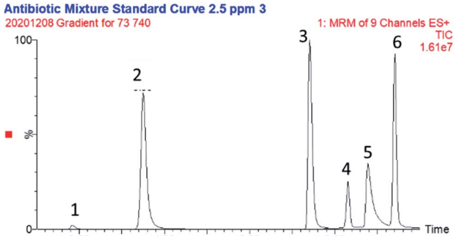


Fig. 1 Overlaid chromatogram (from the first and second acquisition segments) for the separation of the selected antibiotics (1-amoxicillin, 2-metronidazole, 3-trimethoprim, 4-ampicillin, 5-ciprofloxacin, and 6-sulfamethoxazole).

The composition of mobile phase A: 0.01% formic acid in MilliQ and mobile phase B: 0.01% formic acid in methanol for the gradient elution was found appropriate for separating the selected antibiotics. Fig. 1 shows the chromatographic separation of the selected antibiotics. Initially the mobile phase composition of 5% (B) was held for 2 minutes (0–2 min) and was increased to 50% (B) in 9 minutes (2–11 min). It was further increased to 90% (B) in 0.5 minutes (11–11.5 min) and held constant for 2 minutes (11.5–13.5 min). It was decreased back to 5% (B) within 0.5 minutes (13.5–14 min) and equilibrated for another 6 minutes (14–20 min).

Different flow rates were tested (from 0.2 to 0.4 mL min^{-1}), and the optimum one was set to 0.25 mL min^{-1}. Finally, different column temperatures were tested (30 °C and 40 °C). At 40 °C, the peak shapes and chromatographic response improved for all antibiotics except ciprofloxacin, which always had a broad peak.

The injection volume was set at 5 µL. Three distinct data acquisition segments were programmed in the positive mode: the first acquisition segment was from 0 to 4.5 min, the second segment was from 6.5 to 8.0 min and the third segment was from 7.7 to 20 min. The retention times of the studied antibiotics are indicated in Table 2. The retention times for all the compounds were less than 10 min.

3.2 Sample preparation optimization

3.2.1 Recoveries for the filtration step. The filtration of the sample before HPLC injection protects the column from the impacts of particulates and leads to a considerably longer column lifetime. However, during filtration, some compounds can potentially be adsorbed on the filter. Therefore the potential impact of compound adsorption through filtration was evaluated based on compound recovery. The absolute recoveries for the filtration step ranged from 54% to 109% as shown in Fig. 2. Both ciprofloxacin and ciprofloxacin-d8 had low recoveries. Compounds with low absolute recoveries suggest their

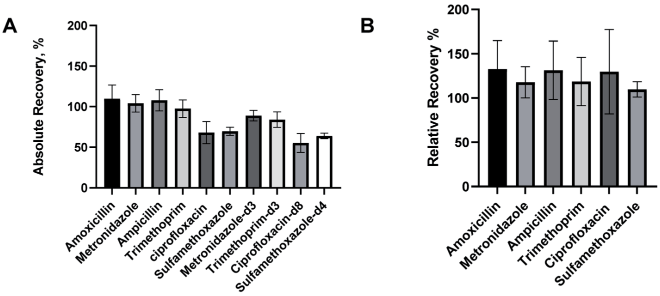


Fig. 2 Recoveries for the filtration step.

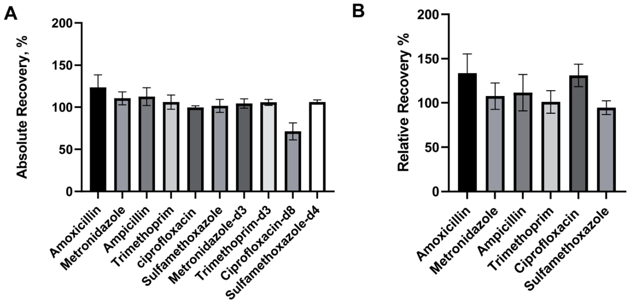


Fig. 3 Recoveries for the evaporation step.

adsorption into the filters. Previous studies showed that ciprofloxacin adsorbs into cellulose filters.[27,28]

3.2.2 Recoveries for the evaporation step. Since the target analytes were extracted with an organic solvent from samples, solvent switching to a highly aqueous solvent to obtain the best chromatographic separation was essential. Solvent switching was performed by evaporating the organic solvent prior to adding the aqueous solvent. The effectiveness of solvent exchange was evaluated based on compound recovery. The absolute recoveries for the evaporation step ranged from 77% to 114%, as indicated in Fig. 3.

3.2.3 Recoveries for the combined extraction, evaporation, and filtration steps. Initially, 10 mL of the extraction solvent was used once to extract the analytes using a sonicator. For some compounds, the obtained absolute recoveries were very low and extremely low (less than 10%) for ciprofloxacin and ciprofloxacin-d8, see Fig. 4A. The use of a tilting shaker instead of a sonicator and performing two extractions significantly improved absolute recoveries as indicated in Fig. 4B.

To improve the recoveries, the composition of formic acid in the extraction solvent was optimised. The extraction was carried

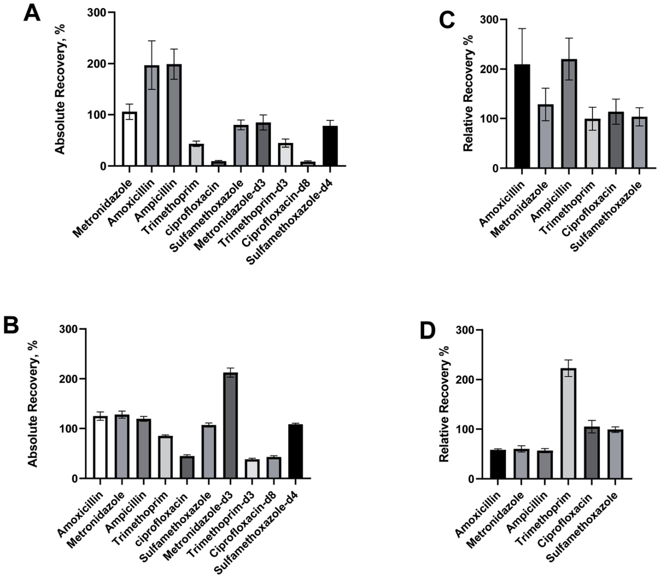


Fig. 4 Recoveries for the combined extraction, evaporation, and filtration steps (A and C extraction using a sonicator and B and D extraction using a tilting shaker).

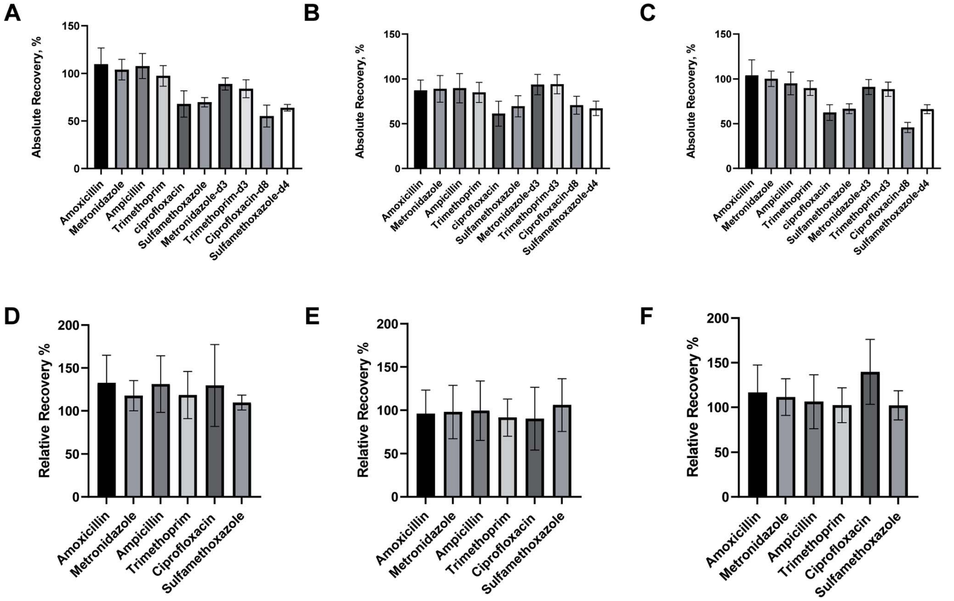


Fig. 5 Effect of formic acid in the extraction solvent (A and D = 0.02%, B and E = 0.05%, and C and F = 0.01%).

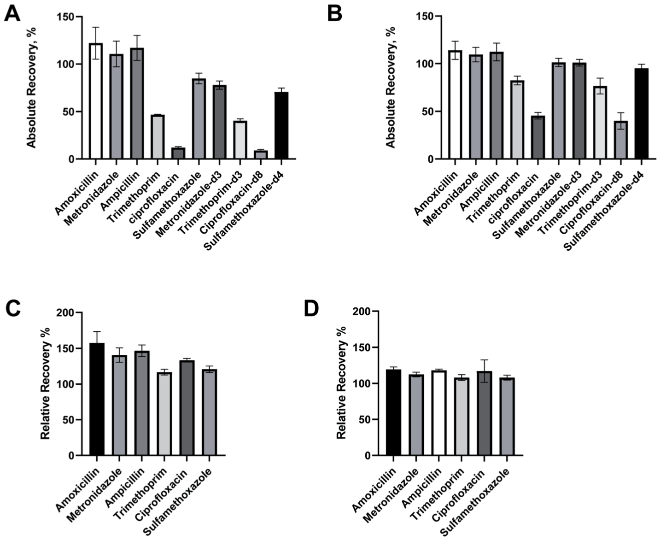


Fig. 6 Recoveries for combined extraction, evaporation with filtration (A and C) and with centrifugation (B and D).

Table 2 Analytical method validation parameters: calibration equation, linearity, limit of detection (LOD), limit of quantification, and precision (% RSD)

Compound	Linear regression equation	r^2	Concentration range (μg mL^{-1})	% RSD		LOD (μg mL^{-1})	LOQ (μg mL^{-1})	Ret. time, min	Matrix factor
				1 μg mL^{-1}	0.01 μg mL^{-1}				
Amoxicillin	$y = 0.5606x - 0.0614$	0.997	0.005–2.5	4.5	15.9	0.036	0.122	1.82	0.90
Metronidazole	$y = 32.477x + 0.0702$	0.996	0.005–1.0	4.8	6.3	0.022	0.072	3.46	0.87
Trimethoprim	$y = 7.4371x + 0.0505$	0.999	0.005–2.5	9.3	7.5	0.012	0.066	7.34	0.92
Ampicillin	$y = 4.9684x - 0.2091$	0.996	0.005–2.5	6.0	14.3	0.046	0.153	8.28	0.82
Ciprofloxacin	$y = 37.198x - 0.1845$	0.999	0.005–2.5	1.6	11.5	0.021	0.072	8.72	0.79
Sulfamethoxazole	$y = 31.435x + 0.4359$	0.998	0.005–2.5	3.1	7.8	0.031	0.104	9.39	0.92

out with varying percentages of formic acid. Fig. 5 shows that 0.05% formic acid in extraction solvent is optimal.

3.2.4 Recoveries for the entire procedure. During the evaporation of the organic solvent, it was observed that some previously dissolved matrix components precipitated out from the sample. Therefore either filtration or centrifugation of the sample was necessary before analysis by LC-MS/MS. The absolute recoveries for the compounds ranged from 9% and 120% when a filtration step was included. On the other hand, the absolute recoveries for the compounds ranged from 45% to 114% when a filtration step was replaced with centrifugation as shown in Fig. 6. Centrifugation indicates improved absolute recoveries for some compounds, suggesting that some compounds are adsorbed into the filters during filtration. With the exception of ciprofloxacin and its internal standard, all other compounds had acceptable recoveries.[29]

3.3 Method validation

3.3.1 Linearity of calibration curves. Typical equations for calibration curves ($n = 3$) are presented in Table 2. The compounds' calibration curves were linear ($r^2 \geq 0.996$) over the range of 0.005–2.5 μg mL^{-1} for all compounds except metronidazole, whose range was 0.005–1 μg mL^{-1}.

3.3.2 Accuracy and precision. The absolute recoveries for the analytes were in the acceptable range from 77% to 114% except for ciprofloxacin (45%). The precision, expressed as relative standard deviation (RSD) at two concentration levels, was between 1.6 and 15.9%, showing good precession with higher variability at the lower tested concentration level.

Table 3 Samples in which analytes were detected[a]

S/N	Sample ID	Concentration, μg g^{-1}
		Metronidazole
1	DARTEMMC6	D
2	DARTEMMC74	D
3	MWAILEDC4	D
4	MWAILEDC5	1.38
5	MWAILEDC7	D
6	MWANYADC4	D
7	NJOMAKTC36	D
8	NJONJOTC21	D

[a] D-detected but below the limit of quantification.

3.3.3 Limit of detection and limit of quantification. The LOD ranged from 0.012 to 0.046 μg mL^{-1}, while the LOQ ranged from 0.066 to 0.153 μg mL^{-1}.

3.3.4 Selectivity. No interfering peaks were observed at the retention times of the analytes and IS.

3.3.5 Matrix effect. The MF values ranged from 0.79 to 0.92, showing that ions were slightly suppressed by the matrix. The obtained matrix factors are slightly on the lower side of the suggested range of 0.85 to 1.15.[22]

4 Application for screening antibiotics in herbal drugs

The method was used to screen 78 powder herbal products purchased in Tanzania for the presence of the selected antibiotics. The products were prescribed for the treatment of bacterial infections such as typhoid and urinary tract infections (UTIs). All samples were prepared according to the optimized sample preparation procedure and screened with the validated HPLC-MS/MS method. To screen and confirm the presence of a target compound in the samples, the retention times and characteristic fragment ions (quantitative ion and qualitative ion) from the sample were compared with those from standards. While metronidazole was detected in eight samples with the highest concentration of 1.38 μg g^{-1}, amoxicillin, ampicillin, trimethoprim, sulfamethoxazole, and ciprofloxacin were not detected in all samples (see Table 3). The results suggest that the developed method is suitable for the confirmation and quantification of adulterants in powdered herbal products.

5 Conclusion

A method was described for the simultaneous screening of six antibiotic adulterants (amoxicillin, ampicillin, metronidazole, ciprofloxacin, sulfamethoxazole, and trimethoprim) in herbal products using HPLC-MS/MS. The developed method showed high sensitivity, accuracy, and precision. The developed method can be used for the simultaneous detection and quantification of the selected antibiotic adulterants in herbal products to ensure product safety and public health. The screening of adulterants in herbal products in Tanzania can be performed using the developed method.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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Chapter 4

Optimization of HPLC–MS/MS Method for Determination of Antimalarial Adulterants in Herbal Products ‡

Christopher J. Mwankuna[1] Feven Kiros[2] Eliapenda E. Mariki[1] Faith P. Mabiki[1] Hamisi M. Malebo[3] Robinson H. Mdegela[4] Bjarne Styrishave[2]

[1] Department of Chemistry and Physics, College of Natural and Applied Sciences, Sokoine University of Agriculture, P.O. Box 3038, Morogoro, Tanzania
[2] Toxicology Laboratory, Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark
[3] UNESCO National Commission of the United Republic of Tanzania, 7 Magogoni Street, P.O. Box 20384, Dar Es Salaam, Tanzania
[4] Department of Veterinary Medicine and Public Health, College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, P.O. Box 3015, Morogoro, Tanzania

* Correspondence: cmwankuna@sua.ac.tz

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ORIGINAL PAPER

Optimization of HPLC–MS/MS method for determination of antimalarial adulterants in herbal products

Christopher J. Mwankuna[1] · **Feven Kiros**[2] · **Eliapenda E. Mariki**[1] · **Faith P. Mabiki**[1] · **Hamisi M. Malebo**[3] · **Robinson H. Mdegela**[4] · **Bjarne Styrishave**[2]

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Abstract

The use of herbal products is booming all over the world because of being believed as safer than conventional drugs and free of side effects. However, there are untrustworthy manufacturers who adulterate herbal products by adding conventional drugs which might eventually lead to microbial resistance and herb-to-drug interactions. There is a need to develop methods for detecting adulterants in herbal products. A high performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) method for simultaneous identification and determination of conventional antimalarials (chloroquine, quinine, sulfadoxine, pyrimethamine, mefloquine, lumefantrine, amodiaquine, artemisinin, dihydroartemisinin, artesunate and artemether) in herbal products was developed. Stable isotopically labelled compounds (artemether-d_3, quindine-d_3, and sulfadoxine-d_3) were used as internal standards (ISs) for quantitative analysis. Extraction of analytes was performed using methanol: water: formic acid (90:10:0.1, v/v) and chromatographic separation was done in a gradient mode using mobile phase A: Ultrapure water containing 0.1% formic acid and 1 mM ammonium formate and mobile phase B: Acetonitrile/methanol (50:50) containing 0.1% formic acid and 1 mM ammonium formate. The calibration curves were linear ($r^2 \geq 0.991$) over the range of 0.001–0.3 μg mL^{-1} for all compounds. The limit of detection (LOD) ranged from 0.002 to 0.02 μg mL^{-1} while the limit of quantification (LOQ) ranged from 0.006 to 0.08 μg mL^{-1}. Accuracy, expressed as recovery of spiked herbal products ranged from 52 to 128%. The precision, expressed as percent relative standard deviation (%RSD) at two concentration levels, ranged from 1.0 to 13.8%. The matrix effect expressed as the matrix factor (MF) ranged from 0.77 to 0.97. The developed method was used to identify and quantify conventional antimalarials in herbal product samples from Tanzania. Ten out of 50 herbal products were found to contain amodiaquine, sulfadoxine, pyrimethamine, mefloquine, dihydroartemisinin, artemether and lumefantrine. The developed method is considered a valuable tool for getting a better understanding of the adulteration of conventional antimalarials in herbal products.

Keywords Conventional drugs · Herbal products · Herbal drugs · Adulteration

Introduction

Herbal products have been used to treat malaria for thousands of years and are the source of some modern antimalarial drugs such as artemisinin and quinine [1–4]. The use of herbal products is booming all over the world because of being believed as safer than conventional drugs and devoid of side effects [5]. However, there are untrustworthy manufacturers who adulterate herbal products by adding conventional drugs to enhance the apparent efficacy claimed on the product's label [6, 7]. The consumption of adulterated drugs may lead to life-threating events caused by microbial resistance and herb-to-drug interactions [8–11]. Therefore,

✉ Christopher J. Mwankuna
cmwankuna@sua.ac.tz

[1] Department of Chemistry and Physics, College of Natural and Applied Sciences, Sokoine University of Agriculture, P.O. Box 3038, Morogoro, Tanzania

[2] Toxicology Laboratory, Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark Universitetsparken 2, DK-2100 Copenhagen, Denmark

[3] UNESCO National Commission of the United Republic of Tanzania, 7 Magogoni Street, P.O. Box 20384, Dar Es Salaam, Tanzania

[4] Department of Veterinary Medicine and Public Health, College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, P.O. Box 3015, Morogoro, Tanzania

there is a need to develop screening methods to control these incidents.

Analytical techniques that have been used for identification and quantification of adulterants in herbal products include thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and capillary electrophoresis (CE) [12–15]. Due to herbal products' complex nature, generally, such techniques cannot be used for high confidence identification [16]. Hyphenated techniques such as gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS) and capillary electrophoresis/mass spectrometry (CE/MS) greatly improve the analytical selectivity and sensitivity and have been used for confirmation purposes. The use of high performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) in multiple reaction monitoring (MRM) mode is increasingly popular and considered the gold standards owing to their very high sensitivity and selectivity in comparison with conventional UV detection [17–20].

Since more than one chemical adulterant may be present in one herbal product, simultaneous detection of adulterants in a single run is efficient [19, 21]. Methods for simultaneous determination of various antimalarials in biological samples and pharmaceutical formulation have been reported [22–30]. However, there are limited studies for simultaneous determination of antimalarial adulterants in herbal products. Also the extent of adulteration of herbal products with conventional antimalarial are not clearly stated in the literature.

The purpose of this study was to develop a multi-analyte method for the detection and determination of antimalarial adulterants in herbal products using simple extraction procedures, HPLC separation, and tandem mass spectrometric detection. To our best knowledge, such a methods has not been reported so far. Chloroquine, piperaquine, quinine, sulfadoxine, pyrimethamine, mefloquine, lumefantrine, amodiaquine, artemisinin, dihydroartemisinin, artesunate and artemether (structures are shown in Table 1), were selected for this study. Since the adulterants may be approved drugs, banned drugs or drug analogues, the selection included the drugs that are currently used for malarial treatment such as artemether and lumefantrine as well as the discontinued drugs such as chloroquine [31, 32].

Experimental

Chemicals, solvents and materials

HPLC grade methanol, acetonitrile and formic acid were purchased from VWR Chemicals (BDH, USA) while

Table 1 Optimized MS/MS parameters for the determination of the selected antimalarials and internal standards

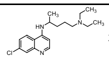
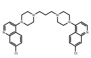
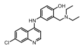
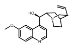
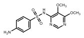
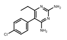
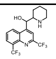
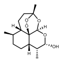
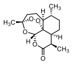
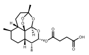
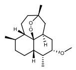
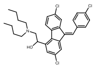
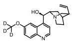
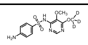
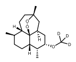
Compound	Chemical Structure	MW (g/mol)	Precursor ion (m/z)	Cone voltage (V)	Product ions^a (m/z)	
					Quantifier	Qualifier
Chloroquine		319.87	320.1 $[M+H]^+$	25	247.0 (24)	142.1 (40)
Piperaquine		535.52	536.22 $[M+H]^+$	50	260.0 (39)	217.0 (46)
Amodiaquine		355.15	356.20 $[M+H]^+$	23	283.0 (45)	255.1 (37)
Quinine		324.40	325.25 $[M+H]^+$	39	160.0 (27)	307.1 (25)
Sulfadoxine		310.30	311.16 $[M+H]^+$	28	156.0 (30)	107.9 (26)
Pyrimethamine		248.70	249.21 $[M+H]^+$	43	233.1 (40)	177.0 (28)
Mefloquinine		378.12	379.22 $[M+H]^+$	39	361.0 (40)	321.0 (31)
Dihydroartemisinin		284.30	302.26 $[M+NH_4]^+$	9	267.1 (8)	163.1 (15)
Artemisinin		282.30	300.26 $[M+NH_4]^+$	10	283.2 (20)	209.0 (10)
Artesunate		384.40	402.31 $[M+NH_4]^+$	12	267.1 (10)	163.1 (21)
Artemether		298.40	316.25 $[M+NH_4]^+$	9	267.1 (9)	163.1 (16)
Lumefantrine		528.94	530.31 $[M+H]^+$	39	348.0 (41)	385.0 (36)
Quinidine-d$_3$		327.44	328.2 $[M+H]^+$	37	163.1 (30)	175.1 (36)
Sulfadoxine-d$_3$		313.35	313.97 $[M+H]^+$	28	156.1 (28)	108.0 (30)
Arthemeter-d$_3$		301.39	319.32 $[M+NH_4]^+$	9	267.1 (9)	163.1 (18)

Table 1 (continued)

MW: molecular weight

[a]Collision energy (in volts) used to obtain each product ion is shown in parentheses

Ammonium formate was purchased from Sigma Aldrich (Glostrup, Denmark). Ultrapure water was obtained using a Milli-Q® UF-Plus apparatus (Millipore Corp., Burlington, MA, USA). Analytical standards of chloroquine diphosphate salt, amodiaquine dihydrochloride dehydrate, quinine, mefloquine hydrochloride, dihydroartemisinin, artesunate, piperaquine tetraphosphate tetrahydrate, pyrimethamine and artemisinin were purchased from Sigma Aldrich (Glostrup, Denmark). Sulfadoxine, artemether, lumefantrine, quinidine-d_3, artemether-d_3, sulfadoxine-d_3 were purchased from Toronto Research Chemicals (TRC, Canada). All analytical standards had purity above 99%. A turmeric herbal product named Solaray Tumeric manufactured by Solaray (Lot number 221579) was bought at pharmacy in Copenhagen, Denmark, and used for the spike recovery studies.

Preparation of standards

Individual stock solutions were prepared by dissolving each analytical standard in methanol, making 1000 µg mL^{-1}, and were stored at − 20 °C. Individual working standards and mixtures were prepared by diluting the stock solutions with 90:10 (A:B, v/v) where mobile phase A contained 0.1% formic acid and 1 mM ammonium formate in water and mobile phase B contained 0.1% formic acid and 1 mM ammonium formate in acetonitrile-methanol (50:50, v/v). For MS optimization, each stock solution was diluted to 10 µg mL^{-1}. The calibration standards were prepared by diluting a mixture of standards at 100 µg mL^{-1} in methanol to 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0 and 6 µg mL^{-1} in mobile phase (90:10 (A:B, v/v)). Each calibration standard contained 2.0 µg of each of the internal standards.

HPLC–MS/MS instrumentation and optimization

The Agilent 1100 liquid chromatography system (Agilent Technologies, Germany) coupled to a Quattro Micro API triple quadrupole Mass Spectrometer (Waters, Micromass Limited, UK) equipped with an electrospray ionization (ESI) interface was used. The LC systems consisted of Degasser, G1379A; Binary pump, G1312A; Autosampler G1313A; Column oven, G1316A; and Diode array detector, G1315B. Separations were performed on a Kinetex analytical column: C18, (2.1 × 75 mm; 2.6 µm) and a 10 mm guard column (Phenomenex, USA). Acquisition and treatment of data were done using Masslynx 4.1 software (Waters Corporation, USA). The nebulizing and collision gases were nitrogen and argon respectively.

The ion source parameters were optimized to obtain adequate and reproducible response for the analytes and internal standards. To determine their fragmentation patterns and select the quantifier and qualifier both direct infusion and flow injection were performed. To select the optimal MS/MS parameters for each analyte, individual standard solutions were prepared at 10 µg mL^{-1} in 90:10 (A:B), where mobile phase A was water and mobile phase B was acetonitrile-methanol (50:50, v/v). Both mobile phases contained 0.1% formic acid and 1 mM ammonium formate as additives. Both the positive and negative ion modes were tested. The precursor ions were selected for collision-induced dissociation to generate characteristic MS/MS spectra for each compound. Two specific precursor-product reaction transitions were selected for each compound. For each compound, the most intense and the next most intense daughter ions were selected for quantification and confirmation, respectively. The Multiple Reaction Monitoring (MRM) mode was employed for data acquisition.

LC optimization for the separation of the investigated antimalarials was done by varying amounts of additives in different mobile phase compositions. For the aqueous phase, ammonium formate/formic acid buffer and Milli-Q with formic acid (FA) and for the organic phase, formic acid in either methanol or acetonitrile or a mixture of methanol and acetonitrile were tested. Varying concentrations of methanol, acetonitrile, formic acid, and formate were investigated. Furthermore, the flow rate and column temperature were optimized to improve chromatographic resolution, peak shapes and reduce total analysis time.

Optimization of sample preparation

Influence of evaporation on recovery of the selected compounds

The selected compounds were extracted from samples using high proportion of organic solvent (methanol: water: formic acid (90: 10: 0.1, v/v). Switching to a highly aqueous solvent to obtain the best chromatographic separation was essential. Solvent switching was performed by evaporating the organic solvent prior to adding the aqueous solvent. The effectiveness of solvent exchange was evaluated based on compound absolute recovery (Eq. 1). The absolute recovery of the target analytes was determined by having one set of samples ($n = 3$) where 200 µL of the 10 µg mL^{-1} standard mixture and 40 µL of the 50 µg mL^{-1} internal standard mixture were made up to 10 mL using the extraction solvent (methanol: water: formic acid (90: 10: 0.1, v/v) and evaporated to 1 mL under a stream of nitrogen at 25 °C. It was then made up to 2 mL with mobile phase mixture (A:B, 95:5, v/v) before analysis. For the second set of samples ($n = 3$), 10 mL of the extraction solvent was evaporated to 1 mL under a stream of nitrogen at 25 °C and spiked with 200 µL of the 10 µg mL^{-1} standard mixture and 40 µL of the 50 µg mL^{-1} internal standard mixture. It was then made up to 2 mL with mobile phase mixture (A:B, 95:5, v/v) before analysis.

Spike recovery for the extraction of selected compounds

A turmeric herbal product bought in Denmark was used to study the recoveries. It was a fine, yellow powdered product packed in capsules of 425 mg each. The spike recoveries were used to assess the optimum conditions for the simultaneous extraction of analytes. The recoveries were determined by a pre- and post-spiked approach where generally, one set of samples ($n = 3$) was pre-spiked with antimalarial standard mixture and post-spiked with internal standard mixture, second set was pre-spiked with internal standard mixture and post-spiked with antimalarial standard mixture and the third set was post-spiked with both the antimalarial standard mixture and internal standard mixture. For each case, the final concentration of analyte was expected to be 1.0 µg mL^{-1}. The absolute recoveries were calculated using Eq. 1. Absolute recoveries of 60–120% were considered acceptable [33].

$$\text{Absolute recovery}(\%) = \frac{\text{Area}_{\text{pre-spiked analyte}} / \text{Area}_{\text{post-spiked IS}}}{\text{Area}_{\text{post-spiked analyte}} / \text{Area}_{\text{post-spiked IS}}} \times 100\% \tag{1}$$

To three aliquots of 250 mg of the sample, 200 µL of a 10 µg mL^{-1} of antimalarial standard mixture was added vortexed and allowed to dry for one hour. It was then extracted twice with 5 mL of methanol: water: formic acid (90: 10: 0.1, v/v) on a tilt shaker for 30 min. It was centrifuge (5300 × g, 5 min) after each extraction. The combined and vortexed extract was evaporated to approximately 1 mL at 25 °C under a stream of nitrogen gas. The sample was transferred to a 2 mL Eppendorf tube, post-spiked with 40 µL of 50 µg mL^{-1} of the internal standard mixture, made up to 2 mL by adding the mobile phase and centrifuged (30000 × g; 5 min). The supernatant was collected for analysis.

A similar experiment was carried out without pre spiking either antimalarial standard mixture or internal standard mixture. However, 200 µL of 10 µg mL^{-1} of antimalarial standard mixture and 40 µL of a 50 µg mL^{-1} of internal standard mixture were post-spiked before making up the extract to 2 mL by adding the mobile phase.

Sample preparation

To 250 mg of a sample, 40 µL of internal standard mixture (50 µg mL^{-1} in methanol) was added, vortexed and allowed to dry for one hour. Samples were extracted twice with 5 mL of methanol: water: formic acid (90: 10: 0.1, v/v) on a tilt shaker for 30 min. To get a clear separation of supernatant and precipitation, the sample was centrifuged (5300 × g, 5 min) for 10 min after each extraction. The extracts were combined and evaporated to approximately 1 mL at 25 °C under a stream of nitrogen gas. The sample was transferred to a 2 mL Eppendorf tube, made up to 2 mL by adding the mobile phase, centrifuged (30000 × g; 5 min) and analysed.

Method validation

The validation process was carried out according to the International Council for Harmonization (ICH) guideline for all the investigated substances [34]. The evaluated validation parameters were selectivity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision and matrix effect.

Selectivity Selectivity of the method was evaluated by comparing the chromatograms obtained from the standard, spiked blank analyte samples and those obtained from the blank samples.

Linearity, limit of detection (LOD) and limit of quantitation (LOQ) Linearity of the calibration curves was established using triplicated standard solutions at nine concentrations ranging from 0.001 to 6 µg mL^{-1}. The curves were constructed by plotting the peak-area ratio of each antimalarial to that of the IS versus the concentration of the drug. Meanwhile analytes that did not have the corresponding internal standards, both external and internal (using the nearby internal standard) calibration approaches were compared. The curves were best fitted using a least-squares linear regression model $y = mx + b$, in which y is the peak area ratio of analyte to IS (for internal calibration approach) or peak area for the external calibration approach, m is the slope of the calibration curve, b is the y-axis intercept of the calibration curve and x is the analyte concentration. The LOD and LOQ were determined based on the standard deviation of the response and the slope using six concentration levels and calculated as provided within the ICH guidelines [34].

Accuracy and precision

The instrumental precision was investigated using neat standards at concentrations of 100 and 300 ng mL^{-1} for all analytes. The accuracy of the method was determined by recovery described in Section titled "Spike recovery for the extraction of selected compounds", whereby the turmeric herbal product was spiked with standards at 1 µg mL^{-1}.

Matrix effect Matrix effect expressed as Matrix Factor (ME) was evaluated to verify whether ion suppression or enhancement due to co-elution of matrix components and analytes existed in the analysis. The peak areas of the analytes and the IS from the post-extraction matrix spike samples were compared to those of the standard solutions in the mobile phase at 1 µg mL^{-1} using Eq. 2. $\mathrm{MF} > 1$, signifies ion enhancement, $\mathrm{MF} < 1$ signifies ion suppression and $\mathrm{MF} = 1$ implies that analytical method is free from matrix effect. Practically, matrix factors below 0.85 (ion suppression) or above 1.15 (ion enhancement) would imply that a matrix effect was present [35].

$$\text{Matrix factor (MF)} = \frac{P_{\text{post-spiked}}}{P_{\text{neat solution}}} \tag{2}$$

Whrere: $P_{\text{post-spiked}}$ is the average analyte peak response of an extracted blank matrix sample spiked with analyte after extraction and $P_{\text{neat solution}}$ is the average analyte peak response of a reference solution at the same nominal concentration.

Results and discussion

HPLC–MS/MS optimization

The optimal source parameters were: capillary voltage of 3.0 kV, exit potential 3 V, RF lens 0.2 V, source temperature 120 °C, desolvation temperature 450 °C, desolvation and cone gas flow rates 650 and 100 L/min respectively. All the compounds responded well in the positive ionization mode. Precursor ions for most of the compounds were molecular ion, $[\mathrm{M+H}]^+$ as observed in previous studies [24, 29]. As also observed in previous studies, artemisinin and its derivatives (dihydroartemisinin, artesunate and artemether) had ammonium derivatives, $[\mathrm{M+NH_4}]^+$, as precursor ions [30, 36–38]. Hence, 1 mM ammonium formate buffer was included in the mobile phase. The optimized compound specific parameters are indicated in Table 1.

The best separation of all the selected compounds was obtained using Mobile phase A: Milli-Q water containing 0.1% formic acid and 1 mM ammonium formate and Mobile phase B: Acetonitrile/methanol (50:50) containing 0.1% formic acid and 1 mM ammonium formate. Separation was achieved using a gradient elution in which the mobile phase

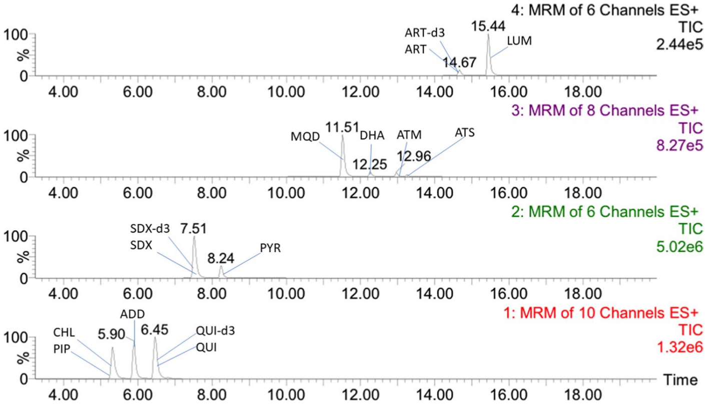


Fig. 1 Chromatogram obtained for the separation of a mixture of antimalarial standards and internal standards (10 µg·mL^{-1}) at the optimum mass parameters and chromatographic conditions. *CHL* Chloroquine, *PIP* Piperaquine, *ADD* Amodiaquine, *QUI* Quinine, *QUI-d₃* Quinidine-d$_3$, *SDX* Sulfadoxine, *SDX-d₃* Sulfadoxine-d$_3$, *PYR* Pyrimethamine, *MQD* Mefloquine, *DHA* Dihydroartemisinin, *ATM* Artemisinin, *ATS* Artesunate, *ART* Artemether, *ART-d₃* Artemether-d$_3$, *LUM* Lumefantrine

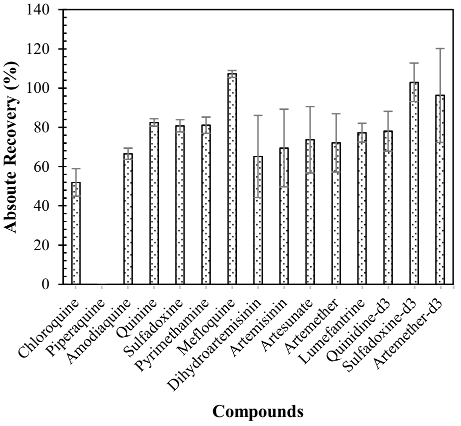


Fig. 2 Influence of evaporation on recovery of the selected compounds and internal standards

composition was increased from 5 to 50% B in the first 9 min and then to 95% B in 6 min and held constant for 2 min. It was then brought back to 5% B in 0.5 min and equilibrated for 2.5 min. The column temperature was 40 °C and the injection volume was 5 µL. Although there was co-elution of chloroquine and piperaquine, their identification was made possible with a mass spectrometer.

The increase in flow rate (from 300 to 400 µL/min) and later the decrease in dwell time (from 250 to 100 ms) led to sharper peaks and a run time of 20 min. Four distinct segments of data acquisition were programmed: the first acquisition segment from 0 to 7.0 min, the second segment was from 7.0 to 9.0 min, the third segment was from 10.0 to 14.0 min and the fourth segment was from 14.0 to 20 min. Figure 1 presents the separation of the selected compounds acquired in the four segments.

Sample preparation optimization

Influence of evaporation on recovery of the selected compounds

Recoveries of the compounds ranged from 52 to 107% with exception of piperaquine (Fig. 2). The recovery of piperaquine was not estimated as it was not detected after evaporation. The loss of piperaquine after evaporation and reconstitution may be attributed to its sticky nature such that it adsorbs to glass [39, 40]. It was then excluded from the subsequent steps of method development.

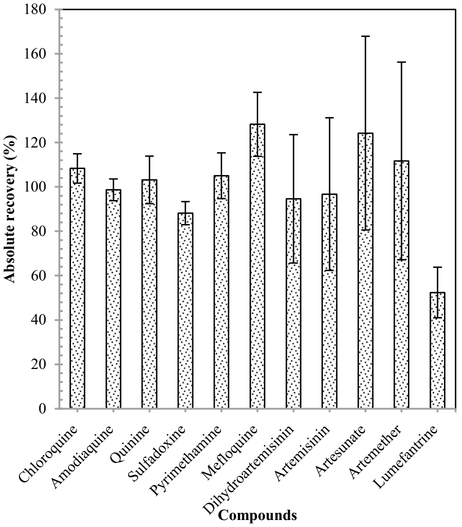


Fig. 3 Spike recovery for the extraction of selected compounds

Spike recovery for the extraction of selected compounds

The recoveries for all the compounds are presented in Fig. 3 and were above 88% except lumefantrine which had a recovery of 52%. Lower recovery for lumefantrine may be attributed to the ion suppression by matrix components as suggested in previous studies [29, 41]. For the rest of the compounds similar recoveries were obtained in previous studied [42]. The recoveries for the analytes were satisfactory [33].

Method validation

Selectivity There were no significant interference peaks of the analytes and internal standards at the retention times.

Linearity, limit of detection (LOD) and limit of quantitation (LOQ) The peak area ratios of calibration standards to the internal standard showed linear relationship with the concentrations of amodiaquine, quinine, sulfadoxine, pyrimethamine, artesunate and artemether. Therefore, internal calibration approach was used for these compounds. On the other hand, the external calibration approach was used for chloroquine, mefloquine, dihydroartemisinin, artemisinin, and lumefantrine since the linear relationship of their peak area ratios to concentration was not good. In all cases, linear relationship ($r^2 \geq 0.991$) was observed in

Table 2 Standard curve range, linearity, LOD and LOQ

Analyte	Regression type	Conc. Range (μg mL^{-1})	Slope	Intercept	r^2	LOD (μg mL^{-1})	LOQ (μg mL^{-1})	% RSD		Matrix factor
								0.1 μg mL^{-1}	0.3 μg mL^{-1}	
Chloroquine	External	0.001–6	24,396	−3968.7	0.997	0.02	0.05	10.9	4.9	0.94
Amodiaquine	Internal	0.001–6	0.223	−0.022	0.991	0.01	0.03	5.8	1.3	0.87
Quinine	Internal	0.001–6	0.063	0.0037	0.992	0.02	0.08	1.0	1.7	0.81
Sulfadoxine	Internal	0.001–6	0.688	−0.001	0.997	0.002	0.007	7.4	4.6	0.80
Pyrimethamine	Internal	0.001–6	0.643	0.016	0.998	0.002	0.006	1.9	2.2	0.77
Mefloquine	External	0.001–6	4893	20.93	0.998	0.002	0.006	6.9	5.5	0.88
Dihydroartemisinin	External	0.001–6	1941	107.6	0.996	0.002	0.006	7.6	8.0	0.97
Artemisinin	External	0.001–6	1628	66.50	0.995	0.002	0.006	11.6	8.7	0.92
Artesunate	Internal	0.001–6	8.55	0.167	0.992	0.002	0.006	12.1	10.2	0.87
Artemether	Internal	0.001–6	313.26	31.56	0.998	0.002	0.006	13.8	11.4	0.77
Lumefantrine	External	0.001–6	9588.9	132.4	0.998	0.002	0.006	5.1	4.8	0.91

a range of 0.001–6.0 μg·mL^{-1} for all the compounds. The limit of detection (LOD) ranged from 0.002 to 0.02 μg mL^{-1} and the limit of quantitation (LOQ) ranged from 0.006 to 0.08 μg mL^{-1} as shown in Table 2.

Accuracy and precision

The percent relative standard deviations (%RSD) for the concentrations of 0.1 and 0.3 μg mL^{-1} were 1.0–13.8 and 1.3–11.4, respectively. The method demonstrated good accuracy between 88 and 128% except for lumefantrine (52%).

Matrix effect The matrix factors ranged from 0.77 to 0.97 showing that ions were slightly suppressed by the matrix as compared to the suggested range of 0.85 to 1.15 [35]. The matrix effects on all the analytes were not significant. In addition, the use of deuterated internal standards, as used in this study, circumvent matrix effects [43–45].

Application for determining conventional antimalarials in herbal products

The validated method was successful used to quantify the conventional antimalarials in 50 herbal product samples from Tanzania. The products were prescribed for the treatment of malaria and other diseases. All samples were prepared according to the optimized sample preparation procedure and analysed using the validated method. For confirmation and determination of a target analyte in the samples, the retention times and characteristic fragment ions (quantitative ion and qualitative ion) from the sample were compared with those from the standard. Ten of the samples were found to contain amodiaquine, sulfadoxine, mefloquine, dihydroartemisinin, artemisinin, artemether and lumefantrine as indicated in Fig. 4.

Conclusions

A selective and sensitive HPLC–MS/MS method for simultaneous identification and quantification of eleven conventional antimalarials (chloroquine, quinine, sulfadoxine, pyrimethamine, mefloquine, lumefantrine, amodiaquine, artemisinin, dihydroartemisinin, artesunate and artemether) in adulterated in herbal products was developed. Amodiaquine, sulfadoxine, pyrimethamine, mefloquine, dihydroartemisinin, artemether and lumefantrine were detected and quantified in 10 herbal product samples from Tanzania. The developed method is suitable for the detection of all the studied antimalarial adulterants in herbal products to monitor the products and safeguard the users.

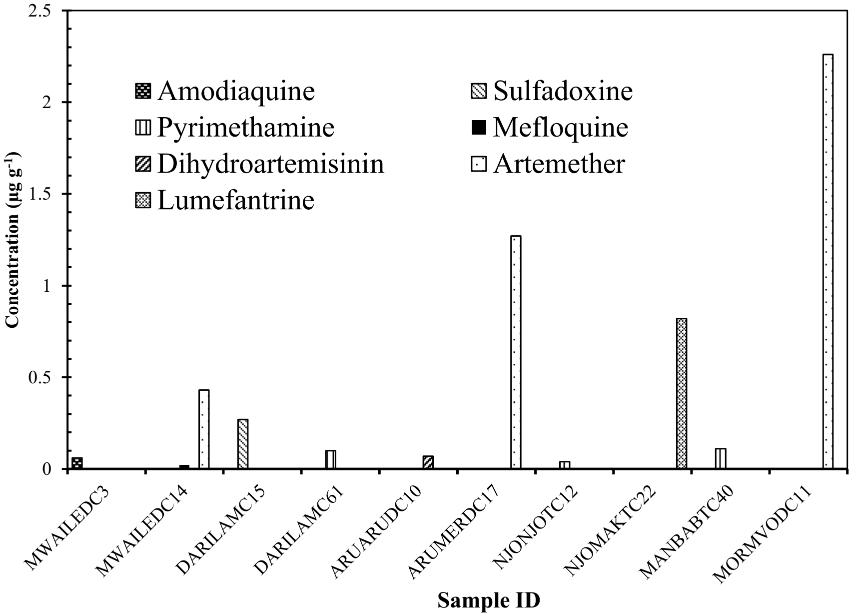


Fig. 4 Herbal product samples found to contain conventional antimalarials

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Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Chapter 5

General Discussion

TLC is a convenient method for screening of samples before using confirmatory techniques. In this study TLC methods were developed and used for preliminary screening of herbal products for the presence of conventional drugs. Two hundred twenty nine (229) herbal products were screened for the presence of trimethoprim, sulfamethoxazole, pyrimethamine, paracetamol, sulfadoxine, metronidazole, sildenafil, lumefantrine, acetyl salicylic acid, diclofenac, ibuprofen and quinine. Trimethoprim, sulfamethoxazole, pyrimethamine, paracetamol, sulfadoxine and metronidazole were among the frequently detected adulterants. Sildenafil and paracetamol have been identified to be frequently added in herbal medicine to enhance the potency and analgesic effects respectively (Pratiwi et al., 2018; Saberi et al., 2018). Half of the products prescribed for pain and bacterial infections contained adulterants. This is attributed to intentional profit making by manufacturers as pain and bacterial infections are the most frequent health problems for which people use herbal products (Ariffin et al., 2021; Chandra et al., 2013; Wirth et al., 2005). Some adulterants appeared in categories not relevant to their claimed indication or purpose. For example, although antidiabetic conventional drugs were not screened in the products, antidiabetic products were found to contain other conventional drugs such as trimethoprim, sulfadoxine, and ibuprofen.

Given the complex nature of herbal products, the combination of high performance liquid chromatography with mass spectrometer is a preferred approach for their analyses as it leads to much more information to deal with complex analytical matrices. A single run method was developed by using HPLC–MS/MS for detection of antibiotic adulterants (amoxicillin, ampicillin, metronidazole, ciprofloxacin, sulfamethoxazole, and trimethoprim) in herbal products. This method is simple in terms of extraction, rapid in terms of less run time, and sensitive in terms of detection and useful for routine monitoring. Similar separation and detection of analytes have been carried out in previous studies (Gros et al., 2013; Metli et al., 2015; Voigt et al., 2020). However, the differences in matrices dictate the optimization of the extraction of analytes and sample clean-up.

The HPLC–MS/MS method for determination of antimalarial adulterants in herbal products was also developed and applied. Precursor ions for most of the compounds (piperaquine, chloroquine, quinine, sulfadoxine, pyrimethamine, mefloquine, lumefantrine, and amodiaquine) were molecular ion, $[M+H]^+$. Artemisinin and its derivatives (dihydroartemisinin, artesunate and artemether) had ammonium derivatives, $[M+NH_4]^+$ as also observed in previous studies (César et al., 2011; Hodel et al., 2009; Ongas et al., 2018; Wiesner et al., 2011; Xing et al., 2006). Extraction of analytes was carried out using methanol:water:formic acid (90:10:0.1, v/v). Switching to a highly aqueous solvent to obtain the best chromatographic separation was essential. Solvent switching was performed by evaporating the organic solvent prior to adding the aqueous solvent. Piperaquine was not recovered after evaporation and this was attributed to sticky nature such that it adsorbs to glass (Kjellin et al., 2014; Mwebaza et al., 2020). It was then excluded from the subsequent steps of method development. The method had satisfactory recoveries, limit of detection (0.002–0.020 $\mu g\ mL^{-1}$) and limit of quantitation (0.006–0.08 $\mu g\ mL^{-1}$) (Venn, 2008). The matrix factors (0.77 – 0.97) indicated slight suppression (Kaewkhao et al., 2019). However, the use of deuterated internal standards, as used in this study, circumvent matrix effects (Hewavitharana, 2011; Smith & Thakur, 2017; Xiao et al., 2012).

Chapter 6
General Conclusions and Recommendations

The importance of method development for detection and quantification of adulterants cannot be underestimated to ensure public health and quality of herbal products. This study successfully developed rapid and simple TLC methods for preliminary screening as well as two HPLC–MS/MS methods for simultaneous confirmation and quantitation of adulterants in herbal products. One HPLC–MS/MS method determines antibiotic adulterants while the other antimalarial adulterants. The developed HPLC–MS/MS methods are simple in terms of extraction, rapid in terms of less run time, and sensitive in terms of detection and useful for routine monitoring.

The methods developed in this study are recommended to the herbal products regulatory authorities to be used for controlling adulteration of herbal products with conventional drugs. Given that, adulterants were detected in herbal products, raising the awareness of the general public on the possibility of consuming herbal products adulterated with conventional drugs is necessary. Development of methods for simultaneously screening of antiimpotency, weight loss and antidiabetic adulterants is also recommended.

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Appendices

Appendix 1: Herbal product samples that were screened using the developed TLC methods

S/n	Trade name	Sample code	Centre type	Therapeutic indications
1.	Shuji	ARUARUCC01	Home based	Chronic malaria
2.	Demaus	ARUARUCC02	Home based	Oedema
3.	Fasha	ARUARUCC03	Home based	Typhoid
4.	Osukuru	ARUARUCC04	Street vendor	Hernia, gas in the stomach.
5.	Oraus	ARUARUCC05	Shop	Skin infections
6.	Nguvu	ARUARUCC06	Shop	Erectile dysfunction
7.	Manaa	ARUARUCC07	Shop	Erectile dysfunction
8.	Nanius	ARUARUCC08	Shop	Erectile dysfunction
9.	Waack	ARUARUDC02	Home based	Malaria and erectile dysfunction.
10.	Dr. Eliud herbal	ARUARUDC04	Home based	Diabetic
11.	Endocrine	ARUARUDC07	Home based	Removes poisons in the body
12.	Ant- nephritis powder	ARUARUDC08	Home based	Urinary tract infection (U.T.I), syphilis, gonorrhoea, removes kidney stone

S/n	Trade name	Sample code	Centre type	Therapeutic indications
13.	Backbone	ARUARUDC09	Home based	All body pains
14.	Mchanatu	ARUARUDC10	Home based	Malaria
15.	Asia	ARUARUDC11	Home based	
16.	Tumbo	ARUARUDC12	Home based	Peptic ulcers
17.	Ushuu	ARUARUDC13	Home based	
18.	Sukari	ARUMERDC04	Street vendor	
19.	Didos	ARUMERDC05	Street vendor	
20.	Malare	ARUMERDC06	Street vendor	Malaria
21.	Jitengeni no 166	ARUMERDC07	Clinic	Typhoid and urinary tract infection (U.T.I)
22.	Jitengeni no 167	ARUMERDC08	Clinic	Malaria and urinary tract infection (U.T.I)
23.	Jitengeni no 168	ARUMERDC09	Street vendor	Erectile dysfunction
24.	Gidon	ARUMERDC10	Street vendor	
25.	Hinia	ARUMERDC12	Street vendor	
26.	Hoboas	ARUMERDC13	Street vendor	
27.	Kikatiti	ARUMERDC16	Home based	Sexual transmitted diseases (STDs)
28.	Fomous	ARUMERDC17	Home based	Malaria

S/n	Trade name	Sample code	Centre type	Therapeutic indications
29.	Pancrease	ARUMERDC18	Home based	Diabetes
30.	Qual	ARUMERDC19	Home based	
31.	Ulcers	ARUMERDC20	Home based	Gastric ulcers.
32.	Ujana	ARUMERDC21	Home based	Ujana
33.	Kuharisha	ARUMERDC22	Home based	Managing diarrhoea
34.	Ngiri	ARUMERDC23	Home based	Hernia
35.	Dawa ya malaria na typhoid	DARILAMC01	Shop	Malaria & typhoid
36.	Disut	DARILAMC11	Street vendor	Malaria
37.	Ujana	DARILAMC12	Shop	Female infertility, body fatigue and boosts immune system
38.	Medica	DARILAMC14	Shop	Diabetes
39.	Moringa oleifea leaves powder	DARILAMC15	Shop	Chronic malaria, bones fluidity, diabetes, raising body immunity.
40.	Mbegu za mchicha	DARILAMC16	Shop	Increase body immunity (CD4), kneel pain, diabetes, TB, hypertension, stroke,

S/n	Trade name	Sample code	Centre type	Therapeutic indications
				sore throats, breast cancer, increase bones mucus
41.	Moringa oleifea leaves powder	DARILAMC18	Shop	Chronic malaria, bones fluidity, diabetes, hardens bones, cancers and raising body immunity.
42.	Avocado seed powder	DARILAMC19	Shop	Removes poison
43.	Tahafif herbs (life natural)	DARILAMC21	Shop	Enhance stamina & energy for healthy and happy life
44.	Tahafif herbs (life natural)	DARILAMC23	Shop	Skin disease
45.	Al-bayanna (cranberry)	DARILAMC24	Shop	Chronic urinary tract infections (U.T.I)
46.	Hailee	DARILAMC25	Street vendor	Malaria
47.	Yapee	DARILAMC26	Street vendor	Typhoid
48.	Juku	DARILAMC27	Street vendor	Erectile dysfunction
49.	Kaine	DARILAMC28	Street vendor	Eliminates waist and backbone pains.

S/n	Trade name	Sample code	Centre type	Therapeutic indications
50.	Mzee wa Mombasa	DARILAMC32	Street vendor	All kinds of body pains.
51.	Samoi	DARILAMC35	Street vendor	Erectile dysfunction
52.	Sainie	DARILAMC37	Street vendor	Typhoid
53.	Topa	DARILAMC38	Street vendor	Erectile dysfunction
54.	Kahaf	DARILAMC39	Shop	Diabetes, BP, swelling of legs, ulcers, gas in the stomach, eliminates cholesterol, body detoxification, malaria, typhoid, body pains, asthma, sharp pains, hiccup, nausea and heartburn
55.	Nimea	DARILAMC40	Shop	Urinary tract infection (U.T.I) diabetes and peptic ulcers
56.	Mdipoli	DARILAMC41	Street vendor	Typhoid
57.	Typhoid	DARILAMC42	Street vendor	Typhoid
58.	Waoa	DARILAMC43	Street vendor	Malaria and typhoid
59.	Chaua	DARILAMC44	Street vendor	Malaria and typhoid
60.	Emius	DARILAMC45	Street vendor	Diabetes
61.	Wechaa	DARILAMC46	Street vendor	Malaria and typhoid

S/n	Trade name	Sample code	Centre type	Therapeutic indications
62.	Alanis	DARILAMC47	Street vendor	Malaria
63.	Yobab	DARILAMC48	Street vendor	Male sexual energy
64.	Tainis	DARILAMC49	Street vendor	Malaria and typhoid.
65.	Ducha	DARILAMC50	Street vendor	Malaria
66.	Kisukari	DARILAMC51	Street vendor	Diabetes
67.	Ngomo	DARILAMC52	Street vendor	Malaria and typhoid.
68.	Msina	DARILAMC53	Street vendor	Malaria
69.	Mwingajini	DARILAMC54	Street vendor	Diabetes
70.	Bulan	DARILAMC55	Street vendor	Malaria and typhoid
71.	Mhagata	DARILAMC56	Street vendor	Malaria
72.	Madakigua	DARILAMC57	Street vendor	Diabetes
73.	Lomn	DARILAMC58	Street vendor	Malaria and typhoid
74.	Mkumbi	DARILAMC59	Street vendor	Malaria
75.	Fahari	DARILAMC60	Street vendor	Malaria and typhoid
76.	Goman	DARILAMC61	Street vendor	Malaria

S/n	Trade name	Sample code	Centre type	Therapeutic indications
77.	Laman	DARILAMC62	Street vendor	Headache, migraine, flu, asthma, ulcers, allergy, dizziness, diabetes, typhoid, waist pains, constipations, swelling of the legs, skin infection, hernia
78.	Ibou Dodoma	DARILAMC66	Shop	Headache, migraine, flu, asthma, ulcers, allergy, dizziness, diabetes, typhoid, waist pains, constipations, swelling of the legs, skin infection, hernia
79.	Dood	DARILAMC69	Shop	
80.	Typhoid and malaria	MANBABDC01	Open market	Typhoid and malaria
81.	Erector	MANBABDC02	Open market	Erectile dysfunction
82.	Yozam	MANBABDC03	Open market	Malaria and typhoid
83.	Kibiti upele	MANBABDC04	Open market	Skin diseases
84.	Mlutuluzu	MANBABDC05	Street vendor	Snake bite.
85.	Mufagaree	MANBABDC13	Home based	Diarrhoea and remove swelling in part of the body.

S/n	Trade name	Sample code	Centre type	Therapeutic indications
86.	Mtumbotumbo	MANBABDC14	Home based	Pain in reproductive system.
87.	Rr	MANBABDC15	Home based	Pain
88.	Mlongoli	MANBABDC16	Home based	Asthma
89.	Pehu	MANBABDC17	Home based	
90.	Mpahi	MANBABDC18	Home based	Pain
91.	Wechaa	MANBABDC20	Home based	All body pains
92.	Aramaroroy	MANBABTC01	Clinic	Pain, goitre, leprosy, swelling of kneel, asthma and heart diseases
93.	Moringa drim spick.	MANBABTC02	Clinic	Erectile dysfunction
94.	Losiasiay.	MANBABTC03	Clinic	Stomach ulcers, increase body immunity (CD4)
95.	Engamay	MANBABTC04	Clinic	Infertility in women, cleanses stomach
96.	Oloisuki	MANBABTC05	Clinic	Allergy, chronic chest pain
97.	Osokonoy	MANBABTC06	Clinic	Sharp pain, pneumonia, chest, asthma.
98.	Loodwa	MANBABTC07	Clinic	Worms, body detoxification, STDs, hernia and pulmonary oedema

S/n	Trade name	Sample code	Centre type	Therapeutic indications
99.	Ngilokita	MANBABTC09	Clinic	Urinary tract infection (U.T.I).
100.	Sukari	MANBABTC11	Clinic	Controls frequent urination, reduce amount of body sugar and cholesterol.
101.	Typhoid	MANBABTC12	Clinic	Chronic typhoid, removes toxins in the stomach and in the blood system.
102.	Ulmac	MANBABTC13	Clinic	Wound healing and pain
103.	Alugo	MANBABTC14	Clinic	Boosts body immunity (CD4), rheumatism and arthritis.
104.	Malaria	MANBABTC15	Clinic	Chronic malaria
105.	Tracking	MANBABTC16	Clinic	Urinary tract infection (U.T.I), and blood detoxification
106.	Rista	MANBABTC17	Clinic	
107.	Komboa	MANBABTC18	Clinic	Malaria
108.	Enous	MANBABTC19	Clinic	
109.	Aloevera powder.	MANBABTC21	Clinic	Stomach diseases, nervous system, urinary disorders like kidney stone

S/n	Trade name	Sample code	Centre type	Therapeutic indications
110.	Glibenclamide tablets	MANBABTC23	Clinic	
111.	Garlic	MANBABTC24	Clinic	More than 70 diseases
112.	Jawabu	MANBABTC34	Shop	Malaria, typhoid, hernia, diabetes
113.	S.H.C	MANBABTC35	Shop	Chronic malaria, typhoid, body pain, hernia, skin diseases and reduce body fats
114.	Ulous	MANBABTC38	Shop	Hernia, waist & backbone pain, stomach-aches, syphilis, pneumonia, legs pain
115.	Malaria	MANBABTC40	Clinic	Malaria
116.	Sukari	MANBABTC42	Clinic	Cancer, remove gas in the stomach and reduce pain in the stomach
117.	Ulmac	MANBABTC43	Clinic	
118.	Amoeba	MANBABTC44	Clinic	Amoeba, U.T.I, and malaria
119.	Fungus	MANBABTC47	Clinic	Fungus
120.	Liver	MANBABTC48	Clinic	Liver disorders
121.	Minyoo	MANBABTC49	Clinic	All kind of worms

S/n	Trade name	Sample code	Centre type	Therapeutic indications
122.	Cancer	MANBABTC50	Clinic	Cancer, remove gas in the stomach and reduce pain in the stomach
123.	Olwesaki	MANSIMDC03	Street vendor	Flu, vomiting and cleanses stomach
124.	Jumbo	MANSIMDC04	Street vendor	Hernia and stomach pain
125.	Olkenasio	MANSIMDC05	Street vendor	Improve body immunity
126.	Olkiloliti	MANSIMDC07	Street vendor	Facilitate digestion of food especially meat
127.	Root of ndemwai	MANSIMDC08	Street vendor	Sharp pain
128.	Olwesuki kwa bibi	MANSIMDC09	Street vendor	Sharp pain
129.	Oltipilikwa	MANSIMDC10	Street vendor	Hernia and nausea.
130.	Olgirai	MANSIMDC11	Street vendor	Bones pains.
131.	Olpinas olmigomi	MANSIMDC12	Street vendor	Erectile dysfunction
132.	Olpukoi	MANSIMDC13	Street vendor	Asthma especially in children.
133.	Olkenasio	MANSIMDC14	Street vendor	Pain in all joints of the body
134.	Olkelelwe	MANSIMDC16	Street vendor	Hernia
135.	Olbukoi	MANSIMDC17	Street vendor	Sharp pain

S/n	Trade name	Sample code	Centre type	Therapeutic indications
136.	Olgera i	MANSIMDC18	Street vendor	Leg pains
137.	Olgera i	MANSIMDC19	Street vendor	Joints pain and bones.
138.	Oldemwai roots.	MANSIMDC20	Street vendor	Waist pain
139.	Olmigomi	MANSIMDC21	Street vendor	Fever, body pain, and increase appetite
140.	Oluisukii (malungulungu)	MANSIMDC22	Street vendor	Cough in children and adults
141.	Olukokola	MANSIMDC23	Street vendor	Joints pain and bones
142.	B.P	NJOMAKDC01	Home	B.P
143.	Yali	NJOMAKDC02	Home	Malaria
144.	Sukari	NJOMAKDC03	Home	Diabetes
145.	Kiboko	NJOMAKDC04	Home	Antibiotic for different diseases
146.	L.K.K.B	NJOMAKTC02	Shop	Peptic ulcers
147.	K.Z	NJOMAKTC03	Shop	Diabetes
148.	M.K.T	NJOMAKTC05	Shop	Boils, rheumatoid bone diseases, muscle pain, cancer
149.	MST	NJOMAKTC06	Shop	Antibiotic

S/n	Trade name	Sample code	Centre type	Therapeutic indications
150.	MP.P.L	NJOMAKTC07	Shop	
151.	L.K.F	NJOMAKTC08	Shop	Typhoid, T.B, hernia, respiratory & inflammatory diseases and pain
152.	HBL:	NJOMAKTC09	Shop	Rheumatoid
153.	L.K.L	NJOMAKTC10	Shop	Cleanses women reproductive system
154.	MY(BP)	NJOMAKTC11	Shop	All heart diseases
155.	Malaria)	NJOMAKTC12	Shop	Malaria
156.	MBZ	NJOMAKTC13	Shop	Cough and diabetes
157.	Tumeric (binzari)	NJOMAKTC14	Shop	Stomach pain
158.	Nourish (paradish)	NJOMAKTC15	Shop	
159.	Mdarasini	NJOMAKTC16	Shop	Ulcers
160.	Kitunguu swaumu	NJOMAKTC17	Shop	B.P
161.	Yovenas	NJOMAKTC18	Shop	Cancer, gastric ulcers, peptic ulcers, lung cancer and diabetes
162.	Hope hemox	NJOMAKTC19	Shop	Diabetes, anaemia, gonorrhoea, syphilis, prostate syndrome

S/n	Trade name	Sample code	Centre type	Therapeutic indications
163.	Hope saragano	NJOMAKTC20	Shop	300 diseases, increase CD4, gastric ulcers
164.	TMB	NJOMAKTC21	Shop	
165.	Gooseberri (amla)	NJOMAKTC22	Shop	Diabetes
166.	PX	NJOMAKTC23	Shop	Erectile dysfunction
167.	Healers	NJOMAKTC25	Shop	Body detoxification
168.	Charcoal powder	NJOMAKTC26	Shop	Gastric ulcers
169.	Fenugreek	NJOMAKTC27	Shop	Hepatitis and diabetes
170.	Herbal care	NJOMAKTC28	Shop	Reproductive system
171.	Nourishment-125	NJOMAKTC29	Shop	
172.	Mhanze 1	NJOMAKTC30	Shop	
173.	Mhanze 2	NJOMAKTC31	Shop	Ulcers
174.	Tagetes erecta (tururu)	NJOMAKTC33	Clinic	Urinary tract infections, mastitis and dry cough
175.	Potato	NJOMAKTC34	Clinic	Urinary tract infections and cancer
176.	Chamolemile	NJOMAKTC35	Clinic	Nervousness and sleepless

S/n	Trade name	Sample code	Centre type	Therapeutic indications
177.	A3	NJOMAKTC36	Clinic	Typhoid, fungus, cancer, rheumatism, diarrhoea, amoeba, malaria
178.	Charcoal mgomba powder	NJOMAKTC37	Clinic	Ulcers, diarrhoea, indigestion and boils
179.	Malaria	NJOMAKTC39	Clinic	Malaria
180.	Zedu	NJOMAKTC40	Street vendor	Malaria
181.	Amma	NJOMAKTC41	Street vendor	Erectile dysfunction
182.	Willia kilaga	NJONJODC01	Home based	Body pains.
183.	Kifua	NJONJODC02	Home based	Typhoid and chest/cough
184.	Ibrahim	NJONJODC04	Home based	Malaria
185.	Kadume	NJONJODC05	Home based	Stomach pains
186.	Flu	NJONJODC06	Home based	Flu and fever
187.	Maumivu	NJONJODC07	Home based	Body pain
188.	Hayao	NJONJODC08	Home based	Hiccup
189.	Mwambutsi	NJONJODC09	Home based	Pneumonia and pains
190.	Tapika	NJONJODC10	Street vendor	Stomach pain and prevent vomiting

S/n	Trade name	Sample code	Centre type	Therapeutic indications
191.	Fever	NJONJODC11	Home based	Stomach and fever
192.	Zandu	NJONJOTC01	Shop	Diaphoretic
193.	Mkombati dawa ya kisukari	NJONJOTC02	Shop	Diabetes, wound healing
194.	Aswel	NJONJOTC05	Shop	Erectile dysfunction
195.	Super	NJONJOTC06	Shop	Erectile dysfunction
196.	Chacoal	NJONJOTC07	Shop	Typhoid
197.	Dawa ya cinamon	NJONJOTC08	Shop	Typhoid
198.	Meth-fenugreek	NJONJOTC09	Shop	Diabetes
199.	C/ment	NJONJOTC10	Shop	Diabetes
200.	Ivigo TMB	NJONJOTC12	Shop	Malaria, gonorrhoea, syphilis, gastro intestinal ulcers
201.	Ivigo viboga	NJONJOTC13	Shop	Hypertension, gastral intestinal ulcer, diabetes, spinal cord and pelvic pain
202.	Ivigo KS	NJONJOTC14	Shop	Detoxification, inflammation, diabetes, tuberculosis, arthritis, rheumatoid

S/n	Trade name	Sample code	Centre type	Therapeutic indications
203.	Ivigo MT	NJONJOTC15	Shop	Typhoid, urinary and respiratory tract infection, gastral intestinal ulcers, diarrhoea
204.	Typhoid	NJONJOTC17	Shop	Antibiotics
205.	MK-subanhari. 2	NJONJOTC19	Shop	Typhoid
206.	Mnyonga pembe	NJONJOTC20	Shop	Menstrual disorders
207.	Mfreta	NJONJOTC21	Shop	Worms, typhoid
208.	Mashine	NJONJOTC22	Shop	Erectile dysfunction
209.	Malaria one	NJONJOTC24	Shop	Malaria
210.	Shaft	NJONJOTC25	Shop	Erectile dysfunction
211.	Asweat	NJONJOTC27	Clinic	Chronic malaria, typhoid, hernia, skin diseases and reduce body fats
212.	Mbasha teeth care	NJONJOTC28	Clinic	Dental problems
213.	Gastro	NJONJOTC29	Clinic	Swelling of stomach and gas in the stomach
214.	MK-subanhari. 2	NJONJOTC30	Clinic	Diabetes

S/n	Trade name	Sample code	Centre type	Therapeutic indications
215.	Cancer leaf powder	NJONJOTC31	Clinic	Cervical cancer, pancreatic cancer, asthma
216.	Avocado compound	NJONJOTC32	Clinic	Diabetes, blood pressure, malaria
217.	Tallary herbal compound	NJONJOTC33	Clinic	Arthritis, allergies, flu
218.	MK-subanhari. 2	NJONJOTC34	Clinic	Diarrhoea, headache, body pains, flu, typhoid
219.	Libido (rijaal)	NJONJOTC35	Clinic	Erectile dysfunction
220.	Tiba	NJONJOTC37	Clinic	
221.	Mkombozi	NJONJOTC38	Clinic	Blood pressure, heart disorders and reduce body weight.
222.	Kingly	NJONJOTC39	Clinic	Stomach pain, gas in the stomach, amoeba, gonorrhoea and waist pain
223.	Kangaroo	NJONJOTC40	Clinic	Frequently urination, UTI, stomach pain, dry cough and dental
224.	Osingwai	NJONJOTC41	Street vendor	Malaria
225.	Tembea	NJONJOTC44	Street vendor	Typhoid

S/n	Trade name	Sample code	Centre type	Therapeutic indications
226.	Kuharisha	NJONJOTC45	Street vendor	Diarrhoea
227.	Tiba	NJONJOTC46	Street vendor	Dysentery
228.		MANBABTC45	Clinic	Removes gas in the stomach
229.	Alovera cream	MANBABTC46	Clinic	Skin infections

Appendix 2: Herbal product samples that were screened for antibiotic adulterants

S/n	Trade name	Sample code	Centre type	Therapeutic indications
1.	Fasha	ARUARUCC03	Home based	Typhoid
2.	Jitengeni no 166	ARUMERDC07	Clinic	Typhoid and urinary tract infection (U.T.I)
3.	Dawa ya malaria na typhoid	DARILAMC01	Shop	Malaria & typhoid
4.	Al-bayanna (cranberry)	DARILAMC24	Shop	Chronic urinary tract infections (U.T.I)
5.	Sainie	DARILAMC37	Street vendor	Typhoid
6.	Nimea	DARILAMC40	Shop	Urinary tract infection (U.T.I) diabetes and peptic ulcers
7.	Mdipoli	DARILAMC41	Street vendor	Typhoid
8.	Typhoid	DARILAMC42	Street vendor	Typhoid
9.	Waoa	DARILAMC43	Street vendor	Malaria and typhoid
10.	Chaua	DARILAMC44	Street vendor	Malaria and typhoid
11.	Wechaa	DARILAMC46	Street vendor	Malaria and typhoid
12.	Tainis	DARILAMC49	Street vendor	Malaria and typhoid.

S/n	Trade name	Sample code	Centre type	Therapeutic indications
13.	Lomn	DARILAMC58	Street vendor	Malaria and typhoid
14.	Fahari	DARILAMC60	Street vendor	Malaria and typhoid
15.	Laman	DARILAMC62	Street vendor	Headache, migraine, flu, asthma, ulcers, allergy, dizziness, diabetes, typhoid, waist pains, constipations, swelling of the legs, skin infection, hernia
16.	Ibou Dodoma	DARILAMC66	Shop	Headache, migraine, flu, asthma, ulcers, allergy, dizziness, diabetes, typhoid, waist pains, constipations, swelling of the legs, skin infection, hernia
17.	Halully herbal medicine	DARKIGMC10	Shop	Malaria typhoid, urinary diseases, gastric ulcers, flu, erectile dysfunction, hernia, diabetes, backbone pain and blood pressure
18.	Ozozo	DARKIGMC15	Street vendor	Malaria, urinary tract infection typhoid and erectile dysfunction
19.	Ash-dau	DARKINMC1	Shop	Hernia, headache, backbone pain, menstrual problems, boosts body

S/n	Trade name	Sample code	Centre type	Therapeutic indications
				immunity (CD4), urinary tract infection, heart diseases and gastric ulcers
20.	Halully herbal medicine	DARTEMMC39	Shop	Malaria typhoid, urinary diseases, gastric ulcers, flu, erectile dysfunction, hernia, diabetes, backbone pain and blood pressure
21.	Zero (u.t.i)	DARTEMMC4	Shop	Urinary trach infection (U.T.I)
22.	Zinga mens power	DARTEMMC48	Shop	Erectile dysfunction, frequently urination
23.	Ngiri	DARTEMMC54	Shop	Hernia
24.	U.T.I	DARTEMMC57	Shop	Urinary tract infections
25.	Halully herbal medicine	DARTEMMC6	Shop	Typhoid, malaria, hernia, diabetes, blood pressure, erectile dysfunction
26.	U.T.I	DARTEMMC61	Shop	Urinary tract infections
27.	Al - homma	DARTEMMC74	Shop	Fever, malaria/ chronic malaria, and typhoid
28.	Jawabu: M	DARTEMMC80	Shop	Malaria, typhoid, hernia, diabetes and body detoxification

S/n	Trade name	Sample code	Centre type	Therapeutic indications
29.	Yemea	DARTEMMC85	Shop	Urinary tract infection (U.T.I)
30.	Venus	KILMOSMC11	Vendor street	Urinary tract infection (U.T.I)
31.	Dubius	KILMOSMC19	Street vendor	Urinary tract infection (U.T.I)
32.	Fisho	KILMOSMC20	Street vendor	Typhoid
33.	Bistus	KILMOSMC26	Street vendor	Urinary tract infection (U.T.I) and typhoid
34.	Yozam	MANBABDC03	Open market	Malaria and typhoid
35.	Tracking	MANBABTC16	Clinic	Urinary tract infection (U.T.I), and blood detoxification
36.	Aloevera powder.	MANBABTC21	Clinic	Stomach diseases, nervous system, urinary disorders like kidney stone
37.	Jawabu	MANBABTC34	Shop	Malaria, typhoid, hernia, diabetes
38.	Amoeba	MANBABTC44	Clinic	Amoeba, U.T.I, and malaria
39.	Al- bayanna (cranberry)	MBEMBECC2	Shop	Chronic Urinary tract infection (U.T.I)
40.	ogoo la shamba lawika mjini no. 2	MBEMBECC5	Shop	Sleeplessness Urinary tract infection (U.T.I) and body detoxification

S/n	Trade name	Sample code	Centre type	Therapeutic indications
41.	Makini mixes poer	MBEMBECC9	Shop	Chronic malaria, typhoid, yellow fever, gonorrhoea, syphilis, waist & backbone pain,
42.	Mujarabu haluli tradition medicine	MORKLSDC10	Shop	Malaria, typhoid, Urinary tract infection (U.T.I), gastric ulcers, hernia diabetes backbone pain
43.	Home	MORKLSDC12	Shop	Chronic malaria, typhoid and Urinary tract infection (U.T.I)
44.	Twalib 1	MORKLSDC39	Home	Urinary tract infections (U.T.I)
45.	U.T.I.	MORKLSDC71	Home based	Urinary tract infections (U.T.I)
46.	Lukwangule	MORMORMC18	Home based	Hepatitis, pneumonia, gonorrhoea, severe diarrhoea, influenza, flu, malaria,
47.	Gotukola	MORMORMC20	Home based	Fatigue, depression, psychiatric disorders, Urinary tract infection (U.T.I), syphilis, dysentery, cholera, leprosy, influenza, elephantiasis, hepatitis, stomach pain, diarrhoea, anaemia,

S/n	Trade name	Sample code	Centre type	Therapeutic indications
				asthma, stomach ulcers, epilepsy, diabetes
48.	Jawabu: M	MORMORMC21	Home based	Malaria, typhoid, hernia, diabetes
49.	Asha	MORMORMC25	Home based	Malaria and typhoid.
50.	Gotukola	MORMORMC27	Home based	Fatigue, depression, psychiatric disorders, Urinary tract infection (U.T.I), syphilis, dysentery, cholera, leprosy, influenza, elephantiasis, hepatitis, stomach pain, diarrhoea, anaemia, asthma, stomach ulcers, epilepsy, diabetes
51.	Masai kikundi i	MORMORMC30	Street vendor	Malaria and typhoid
52.	Dawaau ibtwalu sihri	MORMORMC32	Shop	
53.	Manweva	MORMORMC45	Home based	Improve body immunity
54.	Bunyoro tonic 2	MORMORMC50	Home based	All sexual transmitted diseases
55.	Slina one	MORMORMC51	Home based	Urinary tract infections

S/n	Trade name	Sample code	Centre type	Therapeutic indications
56.	Sama power asili	MORMORMC52	Home based	Malaria, typhoid, U.T.I, stomach pains.
57.	Newton 1	MORMVODC10	Street vendor	Malaria and typhoid
58.	Saitoti 5	MORMVODC17	Street vendor	Urinary tract infections and typhoid
59.	Mzungu 1	MORMVODC7	Street vendor	Malaria and typhoid
60.	Mg -1 -125 (chronic diseases)	MWAILEDC30	Shop	Typhoid, and malaria
61.	Safisha	MWAILEDC4	Clinic	Hernia, reproductive problems and menstrual problems
62.	Komesha	MWAILEDC5	Clinic	Chronic malaria, typhoid, U.T.I, amoeba, gonorrhoea, diabetes, urinary system problems, , backbone pain
63.	Kiboko	MWAILEDC7	Clinic	Haemorrhoids, urinary problems, gonorrhoea and amoeba
64.	Husnus -shufaa powder	MWANYADC12	Street vendor	Chronic malaria and urinary tract infection (U.T.I)
65.	Mbegu za mlonge	MWANYADC10	Street vendor	Diabetes, typhoid, body detoxification, reduce body weight and cholesterol

S/n	Trade name	Sample code	Centre type	Therapeutic indications
66.	Dakika tatu	MWANYADC13	Street vendor	Hernia, headache, chronic fluu, cough, malaria, u.t.i, clean urinary system, pain during urination, stomach and chest pain.
67.	Mseto pori	MWANYADC19	Street vendor	Chronic urinary tract infections (U.T.I), diabetes, , pains during menstruation, miscarriage, chronic malaria, typhoid, gonorrhoea, syphilis, stomach pains headache
68.	Malaria /U.T.I	MWANYADC20	Street vendor	Amoeba, urinary tract infections (U.T.I), malaria
69.	Mkindingi	MWANYADC4	Street vendor	Chronic malaria and hernia
70.	Tagetes erecta (tururu)	NJOMAKTC33	Clinic	Urinary tract infections, mastitis and dry cough
71.	Potato	NJOMAKTC34	Clinic	Urinary tract infections and cancer
72.	A3	NJOMAKTC36	Clinic	Typhoid, fungus, cancer, rheumatism, diarrhoea, amoeba, malaria
73.	MST	NJOMAKTC06	Shop	Antibiotic

S/n	Trade name	Sample code	Centre type	Therapeutic indications
74.	Ivigo MT	NJONJOTC15	Shop	Typhoid, urinary and respiratory tract infection, gastral intestinal ulcers, diarrhoea
75.	Mfreta	NJONJOTC21	Shop	Worms, typhoid
76.	Kangaroo	NJONJOTC40	Clinic	Frequently urination, UTI, stomach pain, dry cough and dental
77.	MP.P.L	NJOMAKTC07	Shop	
78.	L.K.F	NJOMAKTC08	Shop	Typhoid, T.B, hernia, respiratory & inflammatory diseases and pain

Appendix 3: Herbal product samples that were screened for antimalarial adulterants

S/n	Trade name	Sample code	Centre type	Therapeutic indications
1.	Mchanatu	ARUARUDC10	Home based	Malaria
2.	Fomou	ARUMERDC17	Home based	Malaria
3.	Dawa ya malaria na typhoid	DARILAMC01	Shop	Malaria & typhoid
4.	Moringa oleifea leaves	DARILAMC15	Shop	Chronic malaria, diabetes and improves body immunity
5.	Hailee	DARILAMC25	Street vendor	Malaria
6.	Waoa	DARILAMC43	Street vendor	Malaria and typhoid
7.	Chaua	DARILAMC44	Street vendor	Malaria and typhoid
8.	Wechaa	DARILAMC46	Street vendor	Malaria and typhoid

S/n	Trade name	Sample code	Centre type	Therapeutic indications
9.	Tainis	DARILAMC49	Street vendor	Malaria and typhoid.
10.	Lomn	DARILAMC58	Street vendor	Malaria and typhoid
11.	Fahari	DARILAMC60	Street vendor	Malaria and typhoid
12.	Goman	DARILAMC61	Street vendor	Malaria
13.	Halully herbal medicine	DARKIGMC10	Shop	Malaria typhoid, urinary diseases, gastric ulcers, flu, erectile dysfunction, hernia, diabetes, backbone pain and blood pressure
14.	Ozozo	DARKIGMC15	Street vendor	Malaria, urinary tract infection typhoid and erectile dysfunction
15.	Halully herbal medicine	DARTEMMC39	Shop	Malaria typhoid, urinary diseases, gastric ulcers, flu, erectile dysfunction, hernia, diabetes, backbone pain and blood pressure

S/n	Trade name	Sample code	Centre type	Therapeutic indications
16.	Halully herbal medicine	DARTEMMC6	Shop	Typhoid, malaria, hernia, diabetes, blood pressure, erectile dysfunction
17.	Al - homma	DARTEMMC74	Shop	Fever, malaria/ chronic malaria, and typhoid
18.	Jawabu: M	DARTEMMC80	Shop	Malaria, typhoid, hernia, diabetes and body detoxification
19.	Yozam	MANBABDC03	Open market	Malaria and typhoid
20.	Jawabu	MANBABTC34	Shop	Malaria, typhoid, hernia, diabetes
21.	Malaria	MANBABTC40	Clinic	Malaria
22.	Amoeba	MANBABTC44	Clinic	Amoeba, U.T.I, and malaria
23.	Al- bayanna (cranberry)	MBEMBECC2	Shop	Chronic Urinary tract infection (U.T.I)
24.	Mujarabu haluli tradition medicine	MORKLSDC10	Shop	Malaria, typhoid, Urinary tract infection (U.T.I), gastric ulcers, hernia diabetes backbone pain
25.	Home	MORKLSDC12	Shop	Chronic malaria, typhoid and Urinary tract infection (U.T.I)

S/n	Trade name	Sample code	Centre type	Therapeutic indications
26.	Twalib 1	MORKLSDC39	Home	Urinary tract infections (U.T.I)
27.	U.T.I.	MORKLSDC71	Home based	Urinary tract infections (U.T.I)
28.	Jawabu: M	MORMORMC21	Home based	Malaria, typhoid, hernia, diabetes
29.	Asha	MORMORMC25	Home based	Malaria and typhoid.
30.	Masai kikundi i	MORMORMC30	Street vendor	Malaria and typhoid
31.	Ikwizu	MORMORMC37	Shop	Stomach ulcers
32.	Slina one	MORMORMC51	Home based	Urinary tract infections
33.	Sama power asili	MORMORMC52	Home based	Malaria, typhoid, U.T.I, stomach pains.
34.	Saitoti 1	MORMVOD11	Street vendor	Malaria
35.	Newton 1	MORMVODC10	Street vendor	Malaria and typhoid
36.	Saitoti 5	MORMVODC17	Street vendor	Urinary tract infections and typhoid

S/n	Trade name	Sample code	Centre type	Therapeutic indications
37.	Mzungu 1	MORMVODC7	Street vendor	Malaria and typhoid
38.	Maguju	MWAILEDC14	Street vendor	Malaria
39.	Mkalala	MWAILEDC3	Clinic	Gonorrhoea, syphilis, stomach pains, malaria
40.	Mg -1 -125 (chronic diseases)	MWAILEDC30	Shop	Typhoid, and malaria
41.	Mbegu za mlonge	MWANYADC10	Street vendor	Diabetes, typhoid, body detoxification, reduce body weight and cholesterol
42.	Husnus -shufaa powder	MWANYADC12	Street vendor	Chronic malaria and urinary tract infection (U.T.I)
43.	Dakika tatu	MWANYADC13	Street vendor	Hernia, headache, chronic fluu, cough, malaria, u.t.i, clean urinary system, pain during urination, stomach and chest pain.
44.	Malaria /U.T.I	MWANYADC20	Street vendor	Amoeba, urinary tract infections (U.T.I), malaria

S/n	Trade name	Sample code	Centre type	Therapeutic indications
45.	Mkindingi	MWANYADC4	Street vendor	Chronic malaria and hernia
46.	Yali	NJOMADC2	Home based	Malaria
47.	L.K.F	NJOMAKTC08	Shop	Typhoid, T.B, hernia, respiratory & inflammatory diseases and pain
48.	Gooseberri (amla)	NJOMAKTC22	Shop	Diabetes
49.	A3	NJOMAKTC36	Clinic	Typhoid, fungus, cancer, rheumatism, diarrhoea, amoeba, malaria
50.	Ivigo TMB	NJONJOTC12	Shop	Malaria, gonorrhoea, syphilis, gastro intestinal ulcers

Kuhusu Tasnifu hii

Utafiti huu ulifanyika ili kutengeneza mbinu za uchambuzi na kuzitumia katika kubaini dawa za kisasa zilizoongezwa katika dawa zitokanazo na mimea ili kulinda watumiaji na kuimarisha usalama wa dawa zitokanazo na mimea. Mbinu za *thin layer chromatography* na *high performance liquid chromatography–tandem mass spectrometry* zilitengenezwa. Mbinu za *thin layer chromatography* zilizotengenezwa zinaweza kutumika kufanya uchunguzi wa awali wa dawa zitokanazo na mimea kabla ya kuthibitishwa na mbinu zingine. Kwa upande mwingine, utambuzi na ukadiriaji wa vijiuadudu na dawa za malaria katika dawa zitokanazo na mimea vinaweza kufanyika kwa kutumia mbinu zilizotengenezwa za *high performance liquid chromatography–tandem mass spectrometry*. Mbinu za uchambuzi zilizotengenezwa zinaweza kutumika kudhibiti dawa zitokanazo na mimea dhidi ya uchakachuaji wa kuongeza dawa za kisasa.