







RESEARCH ARTICLE

REVISED Comparative study of *in vitro* antimicrobial potential and phytochemicals of some medical plants [version 2; peer review: 1 approved with reservations, 3 not approved]

Previously titled: Comparative study of the *in vitro* phytochemicals and antimicrobial potential of six medicinal plants

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Abstract

Background: Plants in traditional healthcare services in West Africa were selected based on ethnobotanical data for this study. Aqueous and ethanol extracts from these plants' parts were comparatively screened for phytochemicals and *in vitro* antimicrobial activity.





Methods: The antimicrobial activity of five medicinal plants' extracts (aqueous and ethanol) were evaluated against *Proteus mirabilis* (LHC201), *Pseudomonas aeruginosa* (LHC181) and *Aspergillus fumigates* (LUM156) using the agar-well diffusion protocol. Retailed chloramphenicol and griseofulvin were used as positive controls respectively. Phytochemicals and percentage yield were determined by modified standard methods.




Results: The target bacteria showed varied degrees of susceptibility to both aqueous and ethanol extracts. *A. fumigates* was insensitive to the treatments. The ethanol extracts of the sampled plants' parts showed better inhibitory performance against the target bacteria compared to aqueous extracts. Aqueous and ethanol extracts of *Aframomum melegueta*, *Moringa oleifera* and *Cola nitida* showed marginal difference in inhibitory activity with higher inhibition zones observed for the ethanol extracts of *A. melegueta* seed and *M. oleifera* pod against the target bacteria. Phytochemicals composition and density observed in extractants and plants' parts also varied. Phenols were detected in both the aqueous and ethanolic extracts of *C. nitida* and *C. acuminata*, but appeared relatively richer in extracts of *A. melegueta* seeds and *C. albidum* fruits. *C. nitida*, *C. acuminata* and *A. melegueta* extracts were positive for flavonoids which were undetected in *C. albidum* fruits, *M. oleifera* seeds and pod extracts. No single extract had all the phytochemicals assayed.

Conclusions: Screened extracts of medicinal plants' parts used for this study showed promise antibacterial and resource for developing safer pharmaceuticals. Optimization of the antibacterial potential of the extracts for

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Any reports and responses or comments on the article can be found at the end of the article.

commercial exploitation requires further studies. This study has provided information on the antibacterial property of *C. albidum* fruits which was hitherto underutilized for traditional medicine purpose.

Keywords

Antimicrobial activities, Medicinal plants, Phytochemicals, Bacteriostatic, Bacteriocidal

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REVISED Amendments from Version 1

Reviewer reports drew attention to the Reference numbers of target bacterial isolates ATCC21784 (*Proteus mirabilis*) and ATCC27856 (*Pseudomonas mirabilis*). These have been revised as LHC201 (*Proteus mirabilis*) and LHC181 (*Pseudomonas mirabilis*), respectively, in the Abstract and Methods as issued by the University Health Service culture repository.

Reviewers also advised that Voucher numbers be included for the test-medicinal plants used for the study. *Cola acuminata* (UILH/005/2019/703), *Cola nitida* (UILH004/2019/697), *Aframomum melegueta* (UILH1165), *Moringa Oleifera* (UILH001/1275) and *Chrysophyllum albidum* (UILH006/007/2019/1216) (see Methods-second paragraph).

Reviewer reports suggested the introduction of accession number for the fungal isolate (*Aspergillus fumigatus*) used as one of the target organisms in the study. The *Aspergillus fumigatus* was isolated from composted plant materials in a different study and was identified on the basis of microscopic and cultural characteristics using different academic resources for this preliminary investigation. The culture is submitted to the university culture base/bank sequencing and re-validation (Methods).

Karunanidhi *et al.*, 2019 has been used to reflect currency and spread of literature review. On the technical dept. of the methods, the preference for disc technique in susceptibility test expressed by the version 1 report is scientifically debatable and appreciated. Well diffusion method is also listed among the approved routine antimicrobial susceptibility testing methods in clinical laboratory practices. Selection of method is influenced by a range of factors that may include the nature of culture media, test material and target organism. Balouiri *et al.*, 2016 and Karunanidhi *et al.*, 2019 have also employed this method. Ethanol used on crude was 0.1%, while the concentration of crude extract is 200mg/ml instead of 400mg/ml.

The three (3) reviewers called fungal spore suspension concentration (10^5 spores/ml) which is provided in the 'Assessment of antimicrobial activity' section.

The Discussion section has been reorganized in line with reviewer 1's comments.

See referee reports

Introduction

The ethnobotanical use of medicinal plants and their derivatives (essential oil, resins and soluble extracts) in Africa dates back to early civilization (Sofowora, 1993). Herbal formulated medicine and traditional healthcare practice are globally perceived as comparatively cheaper and more widely accessible to most rural and less-privileged populations around the world than synthetic drugs and orthodox medicine respectively (Lawal *et al.*, 2012). Statistics presented by Fabricant & Farnsworth (2001) in the Bulletin of the World Health Organization showed that close to 65% of the world population relied on medicinal plants for their primary healthcare drugs (Eddouks & Ghanimi, 2013). Consequently, it was estimated that about 39% of drugs developed since 1980 have been from natural plants, their derivatives or analogs (Newman & Cragg, 2007; Verpoorte *et al.* (2010). In addition, Ramawa *et al.* (2009) and Verpoorte *et al.*, (2010) noted that approximately 25% of the currently used modern drugs are derived from natural plants, a number largely composed of analgesics (e.g. morphine), cardiotoxic, chemotherapeutics and antimalarials (e.g. quinine and artemisinin).

In recent decades, there have been growing global concerns on the affordability, the rising cost of producing synthetic drugs and accessibility by end users. Furthermore, the complexity involved in assessing their toxicological profile, eliminating their periodic side-effects and episodic efficacy contributed to undermining humans' clinical dependency on synthetic drugs and scale-up their cost of production (Gupta *et al.*, 2016). Other synthetic and semi-synthetic medicines use that has generated concerns in modern healthcare delivery are associated with possible side-effects and growing emergence of spontaneous or build-up biotic resistance (Langdon *et al.*, 2016). Fair & Tor (2014), and Langdon *et al.* (2016) hypothesized that induced microbial resistance to synthetic antibiotics or their analogs involves various ecophysiological mechanisms such as neutralization of antibiosis effect, modulation of binding receptor site, mutation or acquisition of genes coded for antibiotic resistance, and regurgitation of antibiotic chemicals. These have caused a renaissance of herbal screening for chronopharmacologically safer alternative remedies from natural plants and other biogenetic resources (Westh *et al.*, 2004). The clinical and pharmacokinetics sustainability of the efficacy of many synthetic antibiotics, prophylactics and curative drugs is threatened by the growing emergence of multi-drug resistant pathogenic strains (Bandow *et al.*, 2003). This has redirected the focus of pharmacological research into the exploit of natural plants and their allies (ferns, fungi, algae) for safer generic bio-equivalents with parallel or better therapeutic capacity (Rojas *et al.*, 2003; Savoia, 2012). Synthetic drugs use in animal farming also have implications for the global development of organic foods production.

Plant-derived medicine accounted for more than a quarter of today's pharmacopoeia and over US\$3.5 billion in annual export value of pharmaceutical (Eddouks & Ghanimi, 2013). While the global inventory of ethnobotanicals is growing, the catalogue of their bioactive compounds that improves human health is constantly updated. Over 12,000 bioactive metabolites (primary and secondary) and pigments of plant origin with a wide range of biological activities as well as therapeutic values were documented. Osemwegie *et al.* (2014) noted the inadequacy of current botanical data in capturing the global representation of medicinal plants and ethnobotanical knowledge. This was due to possible inaccessibility of plants in remote ecozones or bias against other related plant biota. While the prehistoric and historic knowledge of numerous health-beneficial plants in Africa seems threatened in recent times, their variable use as herbal remedies for treating diverse ailments may predates civilization (Pasewu *et al.*, 2008). This has facilitated the hybridization of both traditional and modern primary healthcare systems in some continents of the world (Eddouks & Ghanimi, 2013).

Aframomum melegueta (Roscoe) K. Schumann (Alligator pepper), *Chrysophyllum albidum* G. Don (Cherry), *Cola nitida* (Vent.) Schott and Endl., *Cola acuminata* (P. Beauv.) Schott and Endl. (Kolanuts), and *Moringa oleifera* Lam. (Moringa) were listed among over 5,000 species of documented medicinal plants reported by Cunningham (1993) and Mahomoodally (2013). These plants except *Chrysophyllum albidum*, based on ethnobotanical

data, were found to be common in many Nigerian cultures and traditional ceremonies (marriage, coronation, invocation) where they feature as masticatory and spiritual materials (George *et al.*, 2018; Idu *et al.*, 2007; Pasewu *et al.*, 2008). They are also ethnopharmacologically valuable in maintaining, preventing and improving health, and in the traditional treatment of different forms of illness in many African nations (Duraipandiyar *et al.*, 2006; Idu *et al.*, 2007; Mahomoodally, 2013). While the data on West African medicinal plants is hardly current, reports are inconsistent on the use of these plants as insecticides, antimicrobial, molluscicides and nematocides across cultures (Odugbemi, 2006). Similarly, the therapeutic scope and potency of biologically active constituents of plants are to a large extent improved by ecosystem factors, methods of extraction of biometabolites, polarity of extraction solvent(s) used and part of plant assayed (Saini *et al.*, 2016; Shitan, 2016). The interactive or counteractive modulation of bioactive compounds in many extracts responsible for inducing therapeutic effectiveness is still a mystery even though ethnomedicine hypothesized that herbal remedies are stable, and safe. This present study aims to compare the *in vitro* antimicrobial potential, phytochemical profile and solubility of the phytometabolome of selected medicinal plants in aqueous and ethanol solvents.

Methods

Materials

Sterile distilled water, ethanol, dilute hydrochloric acid, 0.1% ferric chloride solution, microbiological media, antibiotics, sodium chloride, barium chloride, chloroform, acetic anhydride and H₂SO₄, NaOH were purchased from Ayo-Sigma (ZSA) Chemicals Ltd in Jos, Plateau State, Nigeria.

Pseudomonas aeruginosa (LHC181), *Proteus mirabilis* (LHC201) and *Aspergillus fumigatus* (LUM156) strains were used as target organisms for the study. The cultures of bacterial strains used were obtained from immune-compromised patients of the University Health Centre by personnel of the institution's laboratory. These were preliminarily observed to have antimicrobial resistance. These strains were later sub-cultured and stored in the Microbial Bank of the Microbiology Laboratory, Landmark University, OmuAran. *Aspergillus fumigatus* was obtained from composted plant materials and kept in similar bank as the bacterial strains. It was identified on the basis of microscopic and cultural characteristics using different academic resources (<https://www.aspergillus.org.uk/species/fumigatus>; Klich, 2002). Each microbe was cultured in bijou bottles containing agar slants of nutrient agar (LAB-008) and Sabourad Dextrose Agar (LAB-009).

Preparation of plant materials for extraction

Cola acuminata [Pal. De Beauv.] Schott and Endl. (UILH004/2019/697), *Cola nitida* (Vent.) Schott and Endl. (UILH/005/2019/703), *Aframomum melegueta* (Roscoe) K. Schum (UILH1165), *Moringa oleifera* Lam (UILH/001/1275), and *Chrysophyllum albidum* G. Don (UILH/006/007/2019/1216) were randomly collected from rainforests in the southern belt of Nigeria during the dry season. These medicinal plants that were selected based on existing ethnobotanical data were authenticated using picture books of tropical medicinal plants (Fayaz & Ramachandran, 2015; Mueller & Mechler, 2005). Further

authentication was done at the University of Ilorin Herbarium, Nigeria.

The seeds of plants used for this study were rid of their coat, air-dried for 14 days and then pulverized with a stainless-steel electric blender. This was then sieved with a 325 micron-mesh sieve before storage in labeled, air-tight, sterile universal bottles. The *Moringa*, seeds and pods were also air dried for 5 days before milling while the two species of kola nuts (*Cola nitida* and *Cola acuminata*) were also sorted, air-dried for 14 days, pounded with a mortar and pestle, air-dried again for another 4 days before milling. The powdered form was later stored in air-tight sterile jars and left on the laboratory bench. In the same vein, the fruit pulp and the fruit apicarp of *Chrysophyllum albidum* were manually separated from the seeds, air dried together for 12 days before subjected to similar protocol as described for others.

Preparation of aqueous extracts

A total of 80 g of each of the pulverized plant materials was weighed, poured into a labeled 500-ml conical flask and then soaked with 400 ml distilled water prior to each being vortexed in linear motion for effective extraction using a bench-top reciprocal shaker for 18 h. The preparations were then filtered with a steam-sterilized white handkerchief affixed to a glass filter funnel that drained to labelled 250 ml sterilize conical flasks. The residue from each plant extraction was stored in labelled cylindrical jar and refrigerated while each filtrate while each filtrate was further processed using a rotary evaporator (Model R-205V) at 55°C until a thick concentrate was obtained. This was later transferred into 250 ml beakers and further concentrated on a Water bath at 50°C until a paste was formed (dry crude extract). The paste was later spatulated into freshly labelled sterile universal bottles and weighed (weight of the dry extracts = weight of the universal bottle containing extract – weight of empty universal bottle).

Preparation of ethanol extracts

A similar weight (80 g) of each pulverized plant material was weighed and soaked in 400 ml of 95% ethanol in labelled 500 ml conical flasks. These were vortexed with a bench-top reciprocal shaker (E5850) for a period of 18 h. The mixture was then filtered and processed using similar extraction protocol for aqueous. Aliquots were prepared from the paste for phytochemical and *in vitro* sensitivity assays.

The percentage extraction yield was estimated for both aqueous and ethanol extracts using the formula:

Extraction yield (%) = Weight of dry extract (g) x 100/Weight of sample used for extraction (g)

Where: weight of dry extract is the actual weight of the extracts and the weight of the sample used for the extraction (g) is the initial weight of the samples measured (80 g).

Phytochemical analysis

Phytochemical screening of both the aqueous and ethanol extracts of the different plant materials was done according to

Tiwari *et al.* (2011). The extracts' concentrates were dissolved with distilled water and ethanol (0.1%) respectively to assay saponins, phenolics, flavonoids and terpenoids of each plant extract respectively. 0.10g of each of the plant crude extracts was weighed in a labelled sterile universal bottle, appropriately solubilized with 10 ml of either distilled water for aqueous crude extract or ethanol (0.1%) for the ethanol crude extract. The aliquote were then transferred into clean labelled test-tubes, heated over a Bunsen flame for 3 min, agitated, filtered with Whatmann filter paper (32 mm), cooled, agitated again continuously for 2 min, left to stand for 10 min and observed for froth. In another protocol, phenols, flavonoids and terpenoids were qualitatively investigated using previously described methods (Harborne, 1998). The intensity of color change was used as indicator of richness, observed and rated mildly intense (+), strongly intense (++), extremely intense (+++). Color intensity was visually assessed using an RGB color chart.

Preparation of culture media

A total of 28 g of nutrient agar (NA) and 65 g of Sabouraud dextrose agar (SDA) were each weighed into a sterile conical flask containing 1000 ml of distilled water and mixed vigorously. Each flask was then corked with an absorbent cotton wool stopper after which it was autoclaved for 15 mins at 121°C. This was later allowed to cool to 40°C and dispensed to labelled Petri dishes in a laminar flow chamber where they were allowed to solidify. The bottom of each of the plates was marked into 4 quadrants.

Standardization of test organisms

In this study, the McFarland turbidity standard method, as described by Forbes *et al.* (2007) was used. The 0.5 McFarland standard, which is equivalent to 1.5×10^8 bacteria/ml was prepared according to standard methods (Zapata & Ramirez-Arcos, 2015). Furthermore, normal saline suspensions of the pure culture of each target bacteria were prepared. Turbidity was comparable to the 0.5 McFarland standards by visual determination. Similarly, a serial dilution of *Aspergillus fumigatus* suspension of 10^{-3} diluent containing 10^5 spores/ml was used.

Preparation of positive and negative controls

A concentration of 0.5 mg/ml of chloramphenicol and griseofulvin was prepared, serving as positive controls for antibacterial and antifungal activities, respectively. A solution of 0.85% NaCl was prepared and used as negative control.

Assessment of antimicrobial activity

The agar well diffusion method was used for the antimicrobial assay (Karunanidhi *et al.*, 2019; Balouiri *et al.*, 2016; Murray *et al.*, 2016; Ncube *et al.*, 2008). Previously prepared nutrient agar plates were flooded evenly with 1 ml of 1.5×10^8 bacteria/ml of each bacterial strain and 10^5 spores/ml for the fungus suspension in triplicates. These were left for 5 min after which four 5 mm wells were bored aseptically with a sterile cork-borer into each inoculated agar plate. Next, 80 mg from each of the prepared crude extract was dissolved in 2 ml of sterile distilled water or 0.1% ethanol, as appropriate. 100 µl of each plant extracts was then used to fill 4 equidistant wells using a micropipette. For the controls, 100 µl each of the commercial

antimicrobials (positive control) and normal saline (negative control) solutions was used to fill the agar wells. The plates were all allowed to incubate at 36°C for 24 h for bacteria and room temperature for 48 h for the fungus. These were then observed for zones of inhibition around the wells. Diameters of zones of inhibition were measured in millimeters with a meter rule.

Results

Yields

The ethanol and water used in this study elicited varying solubility capacities of plant phytochemicals. The water extract from the different plants' parts used had the highest yield range (2.13–22.88%) of soluble phytochemicals compared to 95% ethanol extracts' yield range (1.76–22.74%) (Table 1). A marked contrast was observed between the aqueous and ethanol extract yields of *Chrysophyllum albidum* and *Cola nitida* respectively. Ethanol had the least phytochemical yield value of 1.76% for *Moringa oleifera* pod. This was apparently lower than the lowest yield value for the aqueous (2.13%) extract of the same plant material (Table 2).

Phytochemicals

Assessment of the phytochemical profile of each plant extract showed a marked variation in the phytochemical content of each plant material used in this study. Phenol was detected in all the plant materials evaluated, with the exception of *Moringa oleifera* seeds and pods. Saponin, flavonoids and terpenoids showed inconsistent distributions across the various plant extracts studied, with aqueous extracts of *Moringa oleifera* seeds richer in terpenoid compared to the pod extracts (Table 3). Conversely, *Aframomum melegueta* was negative for terpenoids in the two extractants used for this study. Levels of saponin were observed to be negligible except in extracts of *Cola nitida* and *moringa* seeds of both aqueous and ethanol respectively (Table 4).

Table 1. Weight and percentage yield of aqueous extract from the plant samples.

Plant	Weight of extract	% Yield
<i>Cola nitida</i>	8.46	10.58
<i>Cola acuminata</i>	6.31	7.89
<i>Aframomum melegueta</i>	3.17	3.96
<i>Chrysophyllum albidum</i>	18.30	22.88
<i>Moringaoleifera</i> seed	13.44	16.80
<i>Moringaoleifera</i> pod	1.70	2.13

Table 2. Weight and percentage yield of ethanolic extract from the plant samples.

Plant Extract	Weight of extract (g)	% Yield
<i>Cola nitida</i>	18.19	22.74
<i>Cola acuminata</i>	5.11	6.39
<i>Aframomum melegueta</i>	3.17	4.55
<i>Chrysophyllum albidum</i>	4.74	5.93
<i>Moringa oleifera</i> seed	3.37	4.21
<i>Moringa oleifera</i> pod	1.70	1.76

Table 3. Phytoconstituents of the aqueous plant extracts.

Phytochemical/ Extract	<i>C. nitida</i>	<i>C. acuminata</i>	<i>A. melegueta</i> seed	<i>C. albidium</i>	<i>M. oleifera</i> seed	<i>M. oleifera</i> pod
Phenolics	++	+	+	++	-	-
Saponin	++	-	-	-	++	-
Flavonoids	+	+	+	-	-	-
Terpenoids	+	-	-	+	+++	+

-, absent; +, present in low amounts; ++, present in medium amounts; +++, present in high amounts.

Table 4. Phytoconstituents of the ethanolic plant extracts.

Phytochemical/ Extract	<i>C. nitida</i>	<i>C. acuminata</i>	<i>A. melegueta</i> seed	<i>C. albidium</i>	<i>M. oleifera</i> seed	<i>M. oleifera</i> pod
Phenolics	++	+++	+++	+++	-	-
Saponin	++	-	-	-	-	-
Flavonoids	++	++	+++	-	-	-
Terpenoids	+	+	-	-	-	+

-, absent; +, present in low amount; ++, present in medium amounts; +++ present in high amounts.

Antibacterial activity

The plant extracts investigated showed various degrees of antibacterial activity. The observed zone of inhibition from the edge of each well differed from one target organism to the other. Raw data for zones of inhibition are available on OSF (Nwonuma, 2019). All the plant extracts investigated showed mild to average inhibition capacity which is inferior compared to the positive control (Chloramphenicol). Unlike the target bacteria, the fungus (*Aspergillus fumigatus*) is insensitive to the extracts compared to what was observed in the griseofulvin control treatment (Figure 1 & Figure 2). The negative control wells showed no inhibitory activity. Plant extracts derived from *C. nitida*, *C. acuminata*, *A. melegueta* and moringa pods using ethanol solvents showed better inhibitory activity than the aqueous extracts against *Pseudomonas aeruginosa*. In addition, *Pseudomonas aeruginosa* was the most susceptible target bacterium to the plant extracts screened (Figure 2). Conversely, the aqueous extracts of *C. albidium* and moringa seeds showed better inhibitory performance against the target bacteria than the ethanol extracts. A susceptibility contrast was, however, observed for *Proteus mirabilis* treated with the aqueous extracts of *Cola nitida*. Similarly, the aqueous extracts of *Chrysophyllum albidum* had the best inhibition activity against *Pseudomonas aeruginosa* (11.3mm) and *Proteus mirabilis* (9.4 mm) respectively. The highest inhibitory activity were observed for the ethanol extracts of *Cola nitida* (11.8 mm), *Aframomum melegueta* (15.8 mm) and moringa pod (13.4mm) against *Pseudomonas aeruginosa*.

Antifungal activity

Aspergillus fumigatus showed no visible susceptibility to the various plant extracts investigated with no clear inhibition zone.

Discussion

Herbal healthcare practice is an age long tradition that is now assuming a global dimension, attracting interest primarily in

the improvement of the techniques for standardizing herbal prescriptions and production (Venkatasubramanian *et al.*, 2018). In many African traditional healthcare services, herbal remedies prescribed in the form of decoction or infusion in tincture of ethanol or water are without accurate information to end users on expiring dates, dosage, composition, and usage. While this is presently improving among traditional medicine practitioners and local herbs vendors, the issues of proper approach to their scientific quality control, legislation and regulation of herbal drugs use is still a concern to many African nations. The use of herbal knowledge data in the development of pharmaceutical industries and primary public healthcare in many nations of the world is rapidly growing amidst emerging clinical health and therapeutic concerns associated with synthetic drugs use. Pharmacopeias derived from natural herbs is now rife in the global pharmaceutical market and has become a huge investment (Mahomoodally, 2013). The plants selected for this study were indigenous to Nigeria and were all observed to show promise as antibacterial. This observation corroborated already existing ethnomedicinal data that validated their utility in traditional healthcare provisions.

While further studies may be required to ascertain the *in vivo* toxicologically safety, dosage and microbial resistance responses to different herbal medicines, reports have more frequently traced positive pharmacological, biological and toxicological reaction by humans to the compositional dynamics of bioactive secondary metabolites (Ramawa *et al.*, 2009). Results from the study inferred no correlation in antimicrobial activity and phytochemical yields. This may hypothetically suggest that the yields may not be the true reflection of the requisite composites of the extracts nor the phytochemical composition relevant to microbial antibiosis. Consequently, the positively interactive or synergistic and counter-interactive mechanisms responsible for the optimal microbial inhibitory capacity of plants' extracts vary with the biological nature of the target microbes and are not

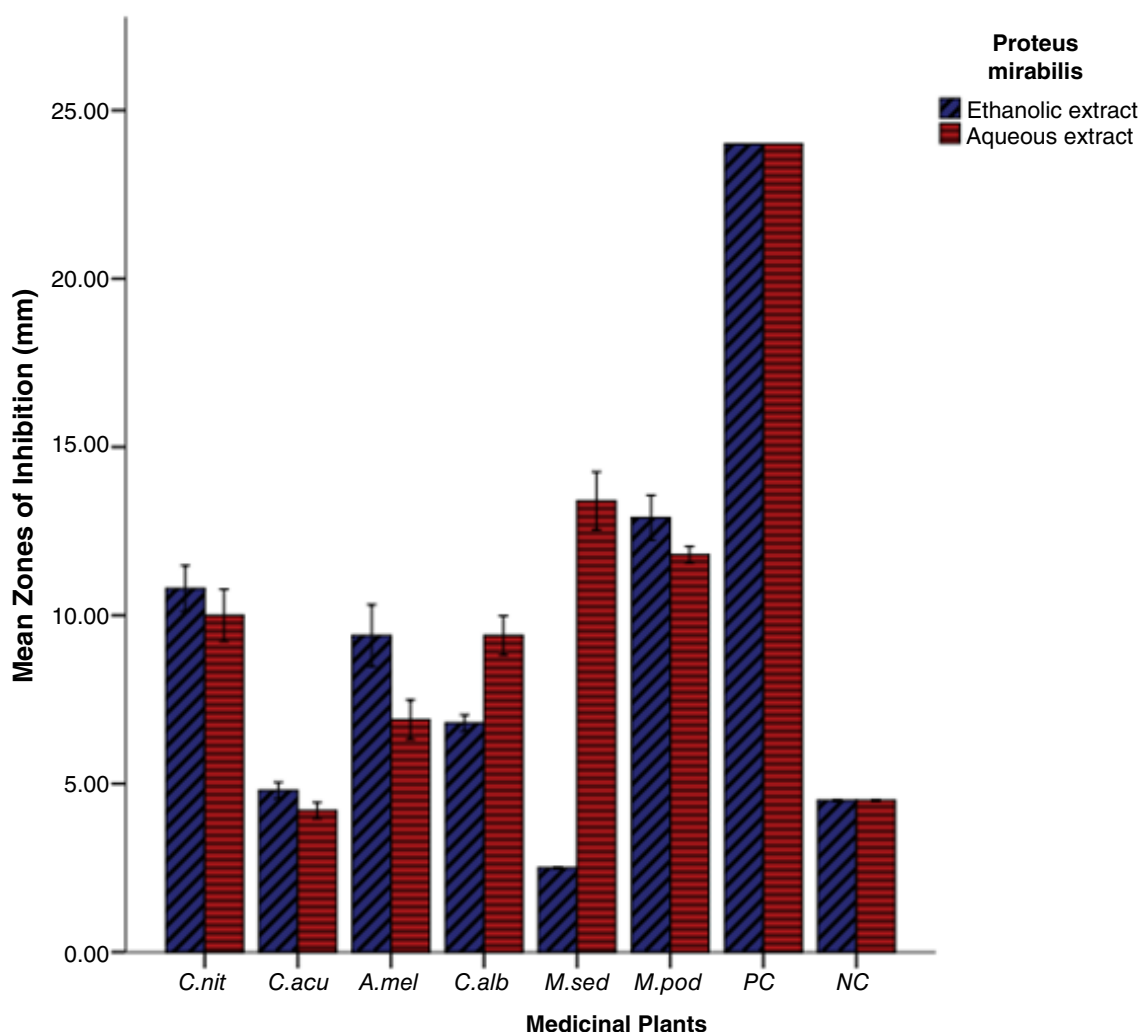


Figure 1. Comparative inhibitory performance (mm \pm SD) of plant extracts on *Proteus mirabilis*.

yet fully understood. It is also philosophical to correlate the inhibitory action of the extracts of the five medicinal plants investigated to the structural modulation of signal transduction and functional groups reaction (Vinoth *et al.*, 2012; Wink, 2015). While the mechanism underlying the solubility preference of secondary metabolites in aqueous or ethanol is not fully understood, it is logical to infer that the aqueous extract selectively solvated more of the non-cytotoxic or bioactively weak secondary metabolites contrary to the ethanol (95%) that theoretical had lower phytochemical composition. Theoretically, ethanol may have the capacity to selectively solvate a consortium of phytochemicals potentially reactive for optimal biologically activities or that afforded a synergic reaction with the best bacteriocin action. This compared to the aqueous could have also accounted for the superior inhibitory performance of ethanol despite the low yield percentage values obtained (Nascimento *et al.*, 2000). It is infrequently noted in literature that the indiscriminate use of herbal tinctures of ethanol and aqueous infusions or decoctions could have histopathological consequence in humans (Fair & Tor, 2014;

Langdon *et al.*, 2016; Osemwegie *et al.*, 2017). Conversely, their oral administrations in healthcare practice in this part of the world remained poorly standardized and without scientific therapeutic protocol. Although every plant materials used for this study were perceived by most local populations to have multi-therapeutic uses in the treatment of numerous ailments (cancer, fever, infections, inflammations, hypertension, diabetics, obesity, dementia, etc.) with no recorded cases of side effect(s) in humans, they showed varying level of systemic potency against the target bacteria. This variation may be linked to the intrinsic or acquired immunity resulting from concomitant exposure to rapidly changing ecological pressures (Soares *et al.*, 2012).

The target microorganisms were observed to be most susceptible to the fruit, seed and pod extracts (ethanol and aqueous) of *Cola nitida*, *Aframomum melegueta* and *Moringa oleifera* respectively. This comparative variation may be attributed to a number of factors including their chronopharmacology, growth history of the plant, physiochemical nature of the solvent extractants used and selection of bioactive metabolites in the extracts

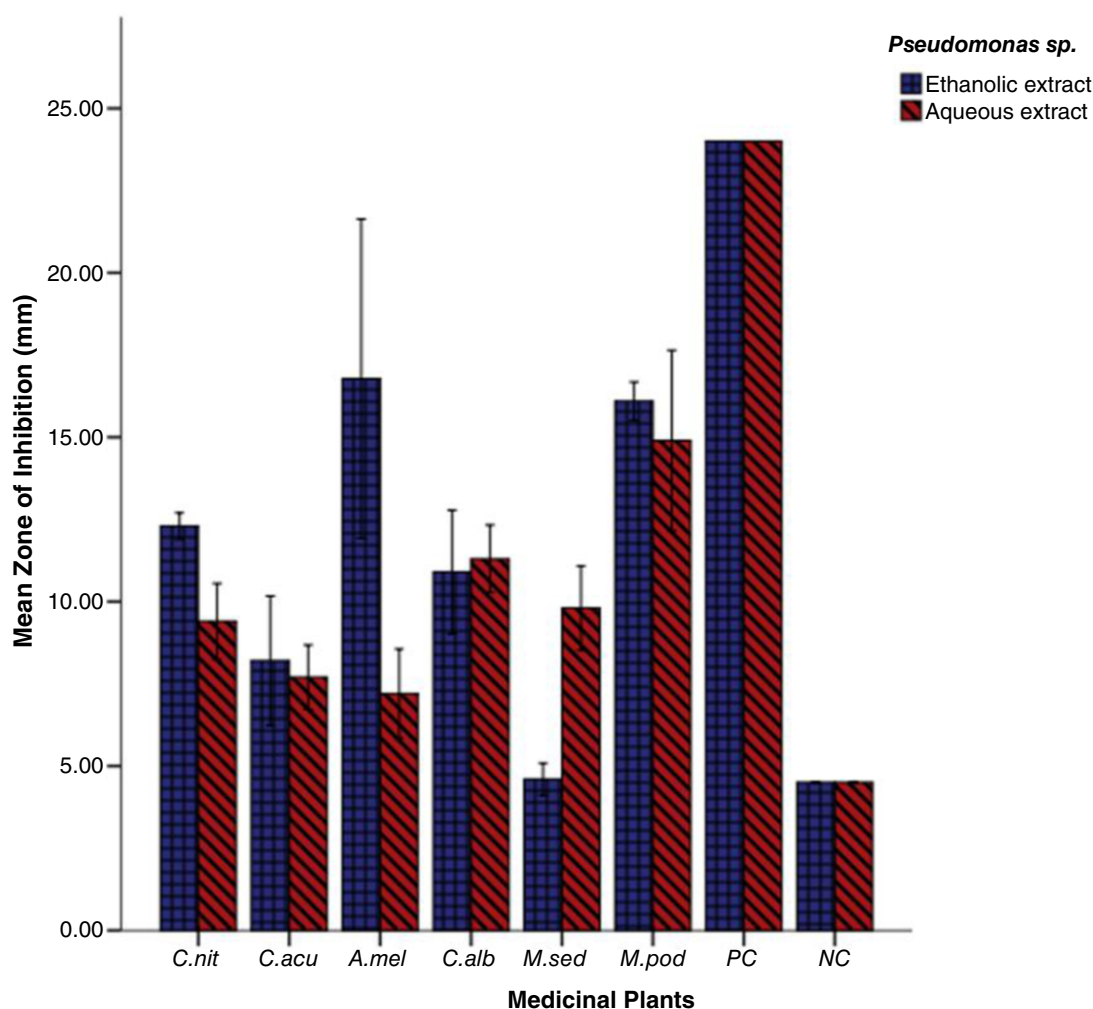


Figure 2. Comparative inhibition performance (mm \pm SD) of plant extracts on *Pseudomonas aeruginosa*. Plants aqueous extracts inhibition performance on *Pseudomonas aeruginosa*; Plants ethanol extracts inhibition performance on *Pseudomonas aeruginosa*.

(Wikaningtyas & Sukandar, 2015). The choice of ethanol and water for this study is consistent with several other antimicrobial studies involving medicinal plants (Ezeifeke *et al.*, 2004). The aqueous extractant was observed to solvate more constituents that did not impact on the inhibitory performance of the extracts of investigated plant materials compared to ethanol (Ahmad *et al.*, 1998; Abu-Shanab *et al.*, 2004; Bacon *et al.*, 2017; Cowan, 1999; Mothana *et al.*, 2010). Phenols and flavonoids were present in most of the extracts phytochemically assayed, they may have influenced positively the observed antimicrobial activity as demonstrated by the results of the ethanolic extracts of *A. melegueta* seeds. The inconsistent pattern of distribution of the phytochemicals may theoretically conflicted such inference. Phenols were detected in all the plant extracts except *Moringa oleifera* seeds. This may have accounted for the observed antimicrobial capacity of the screened extracts (Bukar *et al.*, 2010; Manisha & Vibsha, 2004). The cellular proteins disruption potential and interspecific interactions of phenols or other phytochemicals (flavonoids, tannins, polyketides)

may be assumed to be responsible for the multi-therapeutic uses of the investigated plants. This finding is, however, inconsistent with the report of Saini *et al.* (2016) and Fahal *et al.* (2018), who both observed higher deposits of flavonoids than phenols in the seeds and pods of moringa plant. The difference in the extraction and susceptibility test protocols may have accounted for the contradiction and detection of more of the assayed phytochemicals in the fruit extracts of *Cola nitida*. Furthermore, the fruits of *C. nitida* and *C. acuminata* extracts screened in this study showed low antibacterial activity relative to the other plants' parts. While *Chrysophyllum albidum* was the least ethnobotanically popular of the plant studied (Adewoye *et al.*, 2011; Idu *et al.*, 2007; Okoli & Okere, 2010), its fruit extracts proved to have antibacterial activity that is comparable with the seeds of *A. melegueta*, *Moringa oleifera* seeds and pods. This observation concurred with the study by George *et al.* (2018) and validates *Chrysophyllum albidum* fruits as a health food and an equally valuable pharmacological resource for the development of antibiotics.

Although this study showed that *Pseudomonas aeruginosa* is relatively more sensitive to both crude aqueous and ethanol-based plant extracts than *Proteus mirabilis*, the mechanism underlying the sensitivity reaction remains unclear. In theory, the plant extracts are made up of biochemicals with specific and non-specific modes of actions (cytotoxic and non-cytotoxic) that may compromise the resistance of the target bacteria. The potency and efficacy of the extracts may be the product of the interaction of multiple phytochemicals. Mild sensitivity by *Proteus mirabilis* could hypothetically be as results of induced resistance (Venkatasubramanian *et al.*, 2018; Wink, 2015). Further studies on the isolation and identification of the phytochemical agent(s) responsible for the observed antibacterial activity using High-Performance Liquid Chromatography and Gas Chromatography may be required for better understanding of their mode of action. Target bacteria growth inhibition may also be attributed to a disruption of cell protein biochemistry such as ion channels and pumps, enzyme actions, cytoskeleton function and membrane biochemical modulation (Ekpendu, 1995; Idowu *et al.*, 2006; Wink, 2015). The insensitivity of *Aspergillus fumigatus* may be due to its biological complexity coupled with its innate defensive enzyme mechanism (Osemwegie *et al.*, 2017). While the method adopted for the sensitive test of the fungus may influence the result, the result may be assumed to be derived from fungi long lasting symbiotic relationship with terrestrial plants.

This preliminary study attested to the inherent antibacterial property in all the plants investigated irrespective of the parts used corroborating their prevalence in the Nigeria herbal health-care practice. Similarly, ethanol as an extraction solvent selectively optimized more effectively the expression of antimicrobial

principles in the plants compared to the aqueous solvent. This observation supported the common practice of herbal tincture and offered a scientific basis for the continuous use of ethanol tincture in most local herbal preparations. It is interesting to uncover the potential antibiotic property of *C. albidum* fruits which is hitherto neglected as ethnobotanical and least popular of the five medicinal plants screened. The therapeutic usage of *Moringa oleifera* plants' leaf predominates in ethnomedicine and predates the utility of the other plant parts (e.g. roots, barks, pods, fruits) in this part of the world. In addition, the result opened up a potential utility trajectory for ethanol use in optimizing bioactive composites from natural plants for the production of safe, cheap and alternative antimicrobials, and other pharmaceuticals (Abdul *et al.*, 2010; Abu-Shanab *et al.*, 2004). The promise shown by the five medicinal plants predisposes them to possible exploitation for pharmacopeia (Trease & Evans, 2002).

Data availability

Complete raw data containing the zones of inhibition for each extract are available on OSF. DOI: <https://doi.org/10.17605/OSF.IO/5APVE> (Nwonuma, 2019).

Data are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

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Version 2

Reviewer Report 19 September 2019

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Daljit Singh Arora 

Microbial Technology Laboratory, Department of Microbiology, Guru Nanak Dev University, Amritsar, Punjab, India

The manuscript has been reviewed thoroughly and it was felt that the work is too preliminary and lacks the scientific rigour. There are some points which led to this conclusion:

1. The phytochemical analysis has not been described clearly and satisfactorily for the interpretation and usage by the readers.
2. No quantitative isolation and analysis has been done for the detected phytoconstituents, which does not justify the objective of screening a plant for scientific validation its medicinal values.
3. Material and methods section needs to be rearranged appropriately, where phytochemical analysis should be mentioned after preparation of culture media and standardization of test organisms.
4. Conclusion of the study and its significance not drawn appropriately.
5. Why is saline used as negative control for antimicrobial screening of the ethanolic extracts and not 95% ethanol?
6. Study done against only 2 bacteria and 1 fungus, which is not satisfactory. More test organisms should have been included to scientifically validate the spectrum of the tested plants.
7. In figure 1, legends are not explained. Does not convey the meaning to readers. Also, 5mm wells were prepared in the medium for antimicrobial activity. How is the inhibitory zone of 5mm or less justified if the well size is itself 5mm.
8. Also in figure 1 and 2, as per the title...the mean \pm SD is of how many replicates?.. and to the best of our knowledge ,now a day's SD is not used, instead Standard error is more appropriate.
9. No statistical analysis of the data has been done for comparison of the antimicrobial potential.

10. No MIC studies or other antimicrobial activities have been done to scientifically justify the potential of the given plants. Only Agar diffusion results alone are not validated.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: 1. Novel antimicrobial from plants and endophytic fungi. 2. Enzymology of fungal degradation of Lignin and its biotechnological applications

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Version 1

Reviewer Report 17 April 2019

<https://doi.org/10.5256/f1000research.18691.r45881>

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Patchima Sithisarn

Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand

The concept of the work is very interesting about the antimicrobial activities of medicinal plants in Africa. Though there are some crucial points needed to be clarified scientifically.

1. In the title, declaration of 6 plants, though there are 5 plants. It is needed to clarify that they are different preparations of extracts not different species.

2. It is crucial scientifically to describe the methods e.g. quantification of *A. fumigatus* and its experimental standardization as it is fungi. How the fungi grows and be inhibited by extracts in agar which should be different from other organisms which are bacteria that should not be measure in exact same way.
3. Crucial statistical analysis and validity of the data is required to compare antimicrobial activities of the plants to antibiotic of choices.
4. Phytochemical analysis in the research is qualitative. It may not be used as a referable or validate results in a quality scientific paper.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

No

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Antimicrobial, antiviral targeting in pathogenic zoonotic organisms from phytochemicals.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 19 March 2019

<https://doi.org/10.5256/f1000research.18691.r43814>

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Bhim Pratap Singh

Department of Biotechnology, Mizoram University, Aizawl, Mizoram, India

The work done by Nwonuma et al. is a interesting work but lacking proper execution. I felt that authors should have done the assays at-least till quantitative analysis.

1. Major issue is the use of strain ATCC 21784, which shows as *Rhodococcus* sp. (21784) at ATCC site
(https://www.google.com/search?q=ATCC+21784&rlz=1C1RUCY_enIN705IN705&oq=ATCC+217)
2. Were *Aspergillus fumigatus* using, bad language, language of the manuscript needs revision.
3. *Aspergillus fumigatus*, accession number?

I would suggest a serious revision of the manuscript before accepting for indexing.

Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbial secondary metabolites, traditional medicinal plants, DNA fingerprinting

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 04 February 2019

<https://doi.org/10.5256/f1000research.18691.r43338>

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Armen Trchounian 

Research Institute of Biology, Faculty of Biology, Department of Biochemistry, Microbiology and Biotechnology, Yerevan State University, Yerevan, Armenia

The manuscript is of partial interest, the study is too preliminary and poor and should not be considered for indexing in this journal.

Comments:

1. Abstract: Results are not actually summarized. Conclusions can be altered for the reflection of all content of the article. The presented conclusions are incomprehensible.
2. A voucher specimen must be deposited in a recognized herbarium (collection) in case of plants, or otherwise an appropriate chemical fingerprint is required for future reference.
3. There are no proper negative controls. Particularly sterile distilled water and ethanol should be used as negative controls at the concentrations they present in the final test solution. Taking into account that ethanol has antiseptic properties how the authors can distinguish detected antimicrobial activity as result of ethanol or tested phytochemicals action.
4. In figure 1 there are six columns regarding the inhibition zones of positive control chloramphenicol differing each other against the same bacterial strains. This is not clear: why?
5. Most good journals do not accept agar diffusion studies to determine the antimicrobial activity of plants. Many factors influence the agar diffusion assay for plant extracts and results between different laboratories cannot be compared. Agar diffusion assays may work well for single chemical compounds but not for plant extracts containing compounds with different polarities. Non-polar compounds do not diffuse well into the aqueous agar matrix and this underestimates activity. MIC using serial dilution delivers reproducible results to compare results in different laboratories and only extracts with MICs less than 0.1 mg/ml are considered, as interesting ones. Using crude extracts of plant materials with concentrations above 1000 µg/ml in antimicrobial screening protocols should be avoided, because using high concentrations of plant crude extracts can bring to false positive results (Rios and Recio, 2005). During the current study authors used 400 mg/ml concentration of the extracts in antimicrobial tests, which is too high. The concentrations of positive controls are also too high (5 mg/ml)
6. In the article, it is stated that bacterial test strains (*Pseudomonas aeruginosa* (ATCC27856) and *Proteus mirabilis* (ATCC21784)) were isolated from immune-compromised patients of the University Health Centre and available in Microbial Bank of the Microbiology Laboratory, Landmark University. But the ATCC reference numbers were given to them. Are they available in ATCC's microorganism collection?
7. The discussion must be completely rearranged.
8. Language is poor.

Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbiology, Microbial Biotechnology, Plant Biotechnology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 04 Feb 2019

Osarenkhoe Osemwegie, Landmark University, Omuara, Nigeria

We sincerely thank you for the thorough analytics and comments raised. We have already initiated efforts at addressing the issues of comment and wish to respond to some of the issues we disagreed in your reviewer's comment.

1. *There are no proper negative controls. Particularly sterile distilled water and ethanol should be used as negative controls at the concentrations they present in the final test solution. Taking into account that ethanol has antiseptic properties how the authors can distinguish detected antimicrobial activity as result of ethanol or tested phytochemicals action.*

The method adopted only used ethanol as an extractant of bioactive phytochemicals of the test medicinal plants and not meant for solubilizing the crude for antimicrobial assay. We regret such error and appreciate your pointing it out. Similarly, the extracts were only suspended as crude in distilled water. Therefore, the antiseptic reference of ethanol is not appropriate.

1. *In figure 1 there are six columns regarding the inhibition zones of positive control chloramphenicol differing each other against the same bacterial strains. This is not clear: why?*

The method only affords **one positive and one negative control per treatment**. No inhibition was observed and recorded (suppressed column) for the negative control treatments hence the number of columns presented. Differing reaction by the same strain of bacterium to in vitro antibiotic treatment is a possibility possible linked to undetectable slow differing changes in the local micro-environmental conditions.

1. *Most good journals do not accept agar diffusion studies to determine the antimicrobial activity of plants. Many factors influence the agar diffusion assay for plant extracts and results between different laboratories cannot be compared. Agar diffusion assays may work well for single chemical compounds but not for plant extracts containing compounds with different polarities. Non-polar compounds do not diffuse well into the aqueous agar matrix and this underestimates activity. MIC using serial dilution delivers reproducible results to compare results in different laboratories and only extracts with MICs less than 0.1 mg/ml are considered, as interesting ones. Using crude extracts of plant materials with concentrations above 1000 µg/ml in antimicrobial screening protocols should be avoided, because using high concentrations of plant crude extracts can bring to false positive results (Rios and Recio, 2005). During the current study authors used 400 mg/ml concentration of the extracts in antimicrobial tests, which is too high. The concentrations of positive controls are also too high (5 mg/ml). One 2019 (Arunkumar et al., 2019) reference has been used to reflect currency and spread of literature review. On the technical dept. of the methods, the preference for disc technique in susceptibility test expressed by the version 1 report is scientifically debatable and appreciated. Well diffusion method is also listed among the*

approved routine antimicrobial susceptibility testing methods in clinical laboratory practices. Selection of method is influenced by a range of factors that may include the nature of culture media, test material and target organism. Literature as recent as 2019 also exists that have employed this method (Mounyr et al., 2016 – Journal of Pharmaceutical Analysis; Arunkumar et al., 2019 – Molecules). Ethanol used on crude was 0.1% while the concentration of crude extract is 200mg/ml instead of 400mg/ml. The three (3) reviewers called fungal spore suspension concentration (10^5 spores/ml) which is provided in the Assessment of antimicrobial activity).

We partially concur with you that well diffusion method is becoming antiquated but its ineffectiveness in investigating the antimicrobial property of plant extracts remains debatable and it is still receiving acceptance in reputable journal outlets (Balouiri et al., 2016 – <http://dx.doi.org/10.1016/j.jpha.2015.11.005>). We wish for you to know that the first intent of the study was to prove that all the test plants have antimicrobial activity. We refer you to the last paragraph of the discussion which clearly mentioned that the work is preliminary. The concentration used was set based on the outcome of ethnobotanical survey of the location of study and it still generated the expected result despite disagreeing with Rios and Recio, 2005. All other comments on the voucher number for the test plants, reconstruction of the abstract and the discussion, expressive narration of the results will be addressed. While we do not understand what you mean by the language is poor, we wish to appeal for your guidance in the use of the right language hence we would be glad you return to use the edited version of the article. Thank you.

Competing Interests: No competing interests were disclosed.

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