Journal of Pharmaceutical Research International

17(4): 1-11, 2017; Article no.JPRI.33606
Previously known as British Journal of Pharmaceutical Research
ISSN: 2231-2919, NLM ID: 101631759

Evaluation of an Undocumented Polyherbal (Faradin®) Used for the Treatment of Sickle Cell Disease in West Africa. Part II: Antibacterial Activity and Synergism

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Authors' contributions

This work was carried out in collaboration between all authors. Author MCA initiated the design and execution of the study. Author DAG observed the anecdotal clinical efficacy of the medicine and co-wrote the Institutional Review Board protocol. Author LOF formulated Faradin and provided the medicine and plant materials. Author VM contributed to the planning of the microbiology study. Authors NO, ZE and DA contributed to the phytochemistry and the microbiology. Author TA contributed to the clinical relevance and the microbiology study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2017/33606
Editor(s):
(1) Vijay Ramani, College of Pharmacy, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA.
Reviewers:
(1) Ruth Williams-Hooker, University of Memphis, USA.
(2) Michael P. Okoh, University of Abuja, Nigeria.
(3) Fethi Ben Slama, National Institute of Public Health, Tunisia.
Complete Peer review History: http://www.sciencedomain.org/review-history/19727

Original Research Article

Received 22nd April 2017
Accepted 17th June 2017
Published 27th June 2017

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ABSTRACT

Aim: This study focused on evaluation of the anti-bacterial effects and synergism of Faradin®), a polyherbal complementary alternative medicine (CAM) and anti-sickling agent. It is used for treatment of sickle cell disease (SCD) and co-morbidities (such as infection and inflammation) in some West African countries. It is made up of *Zanthoxylum zanthoxyloides, Alnus glutinosa* and *Alchornea cordofolia, designated, not in order as F, M, and V.*

Methods: Antibacterial susceptibility effects were studied using various weight: weight (w/w) ratios (10:1-1.25:1) of Faradin (or single component extract, F, M and V):water and compared to standard controls - erythromycin and penicillin (5mg/ml). Gram (-) *Pseudomonas aeruginosa* and *E. coli,* and gram (+) *Enterococcus faecalis, Staphylococcus aureus* and *Streptococcus pneumonia microorganisms were utilized.* Well diffusion method was used with pre-prepared Mueller-Hinton agar plates for all species except *S. pneumonia,* for which sheep blood agar plates were employed. Synergism or Fractional Inhibitory Concentration Index (FICI) between Faradin and the antibiotics was calculated using modified minimum inhibitory concentration (MIC) for each microorganism [that was derived using lower concentrations (10:1 – 0.078:1 v/v ratios) in addition to the range used for the susceptibility study], FIC for Faradin, and each antibiotic. FICI of ≤ 0 = synergy, 0.5-1.0 = additive effect; > 1.0 = intermediate effect and values > 4 are suggestive of antagonism.

Results: Faradin, F or M extract had a dose-dependent antibacterial effect, with more antibacterial activity against gram (+) bacteria than gram (-) bacteria. Gram (+) *S. aureus* showed greatest susceptibility followed by *S. pneumoniae* (MICs of 1 mg.ml and 8 mg/ml respectively). The V extract showed no antibacterial activity. Greater than 4.0 FICI values were obtained, except for *P. aeruginosa*, indicative of antagonism between Faradin and the antibiotic controls, and possibly different mechanism of antibacterial action. *P. aeruginosa* had FICI of 0.88, indicative of additive effect.

Conclusions: Polyherbal Faradin showed antibacterial action to certain microorganisms and could be potentially effective against co-morbid infections in sickle cell patients while functioning as an anti-sickling agent.

Keywords: Complementary alternative medicine; polyherbal; Faradin®; anti-sickling agent; antibacterial susceptibility; minimum inhibitory concentration; synergism

ABBREVIATIONS

CAM : Complementary Alternative Medicine
SCD : Sickle Cell Disease
FICI : Fractional Inhibitory Concentration Index
VOC : Vaso-occlusive crises
ACS : Acute Chest Syndrome
ROS : Reactive Oxygen Species
RBCs : Red Blood Cells
WHO : World Health Organization
PBS : Phosphate Buffered Saline
MIC : Minimum Inhibitory Concentration
RIPK3 : Receptor-interacting Serine/threonine Protein Kinase-3
NLR : NOD-like receptor (NLR)

1. INTRODUCTION

1.1 Sickle Cell Disease and Co-Morbid Infections

Sickle cell disease (SCD) is a genetic disease of the erythrocytes in which the β-globin chain of the adult hemoglobin (HbA) produces a mutant form known as sickle hemoglobin (HbS). This is a result of replacement of the polar glutamic acid with non-polar valine at position 6 of the146 amino acid β chain. SCD is manifested by many clinical outcomes that include painful vaso-occlusive crises (VOC), stroke, priapism, pulmonary hypertension, acute chest syndrome (ACS) and chronic organ injuries. Sickled red blood cells, as well as leukocytes, platelets and the vascular endothelium, are elements that obstruct vessels and trigger vaso-occlusive crises [1]. The VOC causes intravascular hemolysis, RBCs rupturing and release of free hemoglobin into the plasma. The free hemoglobin has oxidant and inflammatory effects that results in endothelium dysfunction. The heme, hemoglobin and reactive oxygen species (ROS) then results in oxidative stress, decrease in vasodilator nitric oxide (NO) and further inflammation. It has also been recently reported that receptor-interacting serine/threonine protein kinase-3 (RIPK3) enzyme is involved in the regulation of inflammatory responses via activation of NOD-like receptor (NLRP3) [2,3]. The result of the inflammation process in the spleen is obstruction of cells leading to the

infarction of the organ or shrinkage called asplenia in children under five years. Splenic changes in SCD patients have been well documented [4,5]. The subsequent loss of spleen function leads to susceptibility to bacterial infections, especially pneumococcal infections [6]. Therefore, for effective treatment of SCD, the therapeutic agent or complimentary alternative medicine (CAM) or supplement should possess anti-infective, anti-sickling and anti-inflammatory properties.

1.2 Epidemiology

At least 12 million individuals worldwide in Africa, Middle Eastern Arab countries, Mediterranean, Europe, Asia and United States have SCD [7-9]. One third of the 12 million (approximately 4 million) people with the disease reside in Nigeria [5]. SCD contributes to 16% of under-five mortality in West African countries [7]. In addition, according to the Centers for Disease Control and Prevention (CDC), 1 in 12 African Americans are affected with the most severe form of sickle cell anemia and 90,000 to 100,000 Americans are affected with sickle cell disease [10]. For children under five, bacterial infections often accompany SCD. The need, therefore, for complementary alternative medicines that are effective and affordable for management of the disease and co-morbid infections would be beneficial.

1.3 Complementary Alternative Medicine

The use of CAM or supplements in the treatment or management of certain diseases is gaining a lot of attention as more research is conducted into CAM. According to World Health organization (WHO) report, millions of people around the world rely on traditional and CAM and practitioners as the main or only source of health care [11]. Some neglected diseases such as SCD have limited number of allopathic therapeutics for treatment and management of the disease state. The use of natural products as supplements for SCD is common especially in the regions of the world where the disease is most prevalent. Many of these regions rely on traditional medicines as they are readily available and relatively cheap compared to chemically synthesized drugs. Examples of such geographical areas are sub-Saharan Africa, India, Middle Eastern Arab and Mediterranean countries.

A number of CAMs currently used in Africa for SCD include Niprisan®, Dioscovite, Hildi, Sicklervite and Faradin®. Faradin®, is a liquid polyherbal (three herbal plants) that have been found anecdotally to be effective for management of SCD (and with no reported toxicity) in Nigeria and few other West African countries. However, very little investigation has been conducted or published on Faradin [12]. It was approved by the Nigerian Agency for Food and Drug Administration and Control (NAFDAC) as a supplement (Certificate number 4-0077L). The components on the label of Faradin® are *Zanthoxylum zanthoxyloides, Alnus glutinosa and Alchornea cordofolia*. Adeyeye et al. discussed in an earlier study the bioactives and medicinal properties of each [13].

Knowing that the components of Faradin have been reported to have many secondary metabolites with antibacterial effects as stated above, it becomes necessary to study the polyherbal on which there has been little investigation or reports in literature [12]. Therefore, the objectives of the study are to investigate the antibacterial properties of Faradin® and extracts of the single components and compare these with standard antibiotics - erythromycin and penicillin, in an attempt to know if there is any synergism between Faradin® and standard antibiotics.

2. MATERIALS AND METHODS

2.1 Materials

The manufacturer, Atipo Ventures of Nigeria, supplied the commercial Faradin liquid medicine and the other individual extract components. The component plants were first collected in 1995 and authenticated at the National Institute for Pharmaceutical Research and Development, Nigeria. The standard antibiotics and erythromycin and other chemicals were purchased from Sigma-Aldrich (USA). Penicillin VK, already prepared Mueller Hinton medium agar plates, tryptic soy agar with 5% sheep blood plates and the bacterial organisms *E. coli C 600, and* microkwik cultures of *S. aureus, P. aeruginosa, E. faecalis and S. pneumonia* were purchased from Carolina Biological Supply, Burlington, NC.

2.2 Methods

2.2.1 Anti-bacterial susceptibility test

2.2.1.1 Antibacterial action of single extract or faradin

To test susceptibility, Gram (-) bacteria strains, *P.* aeruginosa and *E. Coli,* and three strains of

gram (+) bacteria – *S. aureus, E. faecalis* and *S. pneumoniae* were used. The microorganisms were grown in nutrient broth at 37°C for 18 h and standardized using normal saline to turbidity of 0.5 McFarland standards (10^8 cfu/cm^3). Well diffusion method was used with pre-made Mueller-Hinton agar plates for all the bacterial strains except for *S. pneumonia* for which sheep blood agar plate was used. Four equidistant holes were made with a sterile cork borer (6 mm diameter) followed by adding a drop of molten agar to each hole and allowing it to solidify to seal well bottoms and to avoid seepage of extract. Prior to use, the single component extract or Faradin was de-alcoholized using the rotary evaporator. 200 µl of varied w/w concentrations (range of 10:1 – 1.25:1) of Faradin or the single extracts (coded as F, M and V) were screened against penicillin VK or erythromycin controls. The agar plate samples were then incubated for 24 h at 37°C. Triplicate tests were carried out for each concentration and the mean diameters of zones of inhibition were calculated.

2.2.1.2 Determination of minimum inhibitory concentration (MIC)

The Karuppusamy and Rajasekaran [14] method was used with sterile 96 well plates. Each well contained 175 µl of Faradin or single component extract in the concentrations stated above and 20 µl of a bacterial suspension. The plates wrapped loosely using cling film to ensure that the bacteria did not become dehydrated. Erythromycin or Penicillin VK was used as the standard test drugs. The plates were incubated at 37°C for 12 hrs followed by addition of 5 µl of resazurin (1 mg/ml) to each well and further incubation for 5 h at 37°C. Broth, resazurin solution and 0.5x phosphate buffered saline (PBS) were used as negative controls. The color change was observed visually and with a plate reader. The change of color from purple to pink or colorless would be an indication for the presence of bacterial growth. The lowest concentration at which there was no color change is regarded as the MIC value.

2.2.1.3 Modified minimum inhibitory concentrations (MIC)

Due to the intense brown color of Faradin, the resazurin-based MIC color-indicative method that showed purple color change as a signal to antibacterial effect was difficult to determine. Therefore, an alternate MIC method was used. A well diffusion method was employed with further microdilution of Faradin using Faradin:water weight ratios (range of 10:1 – 0.01:1) as referred to earlier in the susceptibility test above. Specifically, 0.625:1, 0.313:1; 0.156:1; 0.078:1; 0.039:1; 0.020:1 and 0.010:1 (weight ratios) concentrations of Faradin were added to those used for the antibacterial screening. Antibiotic erythromycin or penicillin (5 mg/ml) was used as the controls. The concentration at which the minimum inhibition was observed, or after which there was no inhibition was taken as the MIC.

2.2.2 Synergism of faradin and standard antibiotic studies

Using the modified MIC method, synergism or interaction between Faradin and the antibiotics - erythromycin or penicillin was investigated and the Fractional Inhibitory Concentration Index (FICI) determined using equation below [15].

$$FICI = FICA + FICB = \frac{[Faradin]}{MIC_{Faradin}} + \frac{[Antibiotic]}{MIC_{Antibiotic}}$$

FICA or FICB is the fractional inhibitory concentration of Faradin or the antibiotic, i.e. penicillin or erythromycin. FICI is the sum of the ratio of the Faradin (or antibiotic) concentration and MIC for Faradin or the antibiotic.

[Faradin] or [Antibiotic] is the concentration of Faradin or the antibiotic in mg/ml. MIC for Faradin or either antibiotic, determined from the modified well diffusion method, was used for the calculation.

FICI of ≤ 0.5 is indicative of synergy. FICI values between 0.5-1.0 - indicate additive effect. Values > 1.0-4 reveals an intermediate effect. Values of > 4 are suggestive of antagonism or non-interaction.

2.3 Statistical Analysis

The statistical analysis for minimum inhibitory concentration (MIC) for Faradin were carried out using analysis of variance (ANOVA) single factor with P value < 0.05.

3. RESULTS AND DISCUSSION

3.1 Anti-bacterial Susceptibility Test

3.1.1 Anti-bacterial action of single extracts

The F and M extract displayed a dose-dependent susceptibility of *E. coli, P. aeruginosa, E.*

faecalis, S. aureus and *S. pneumonia* species over the 10:1 – 1.25:1 v/v extract:water ratios when compared to penicillin or erythromycin. However, F or M extract showed more activity against gram (+) bacteria *S. aureus and E. faecalis* compared to gram (-) bacteria *E. coli, P. aeruginosa*. In general, F extract showed more activity. Although it is a gram (+) bacterium, *S. pneumonia* was not as sensitive to either F or M extract. Extract V displayed no antibacterial action compared to either penicillin or erythromycin. Typical profiles are represented in the activity compared with penicillin (Figs. 1-3).

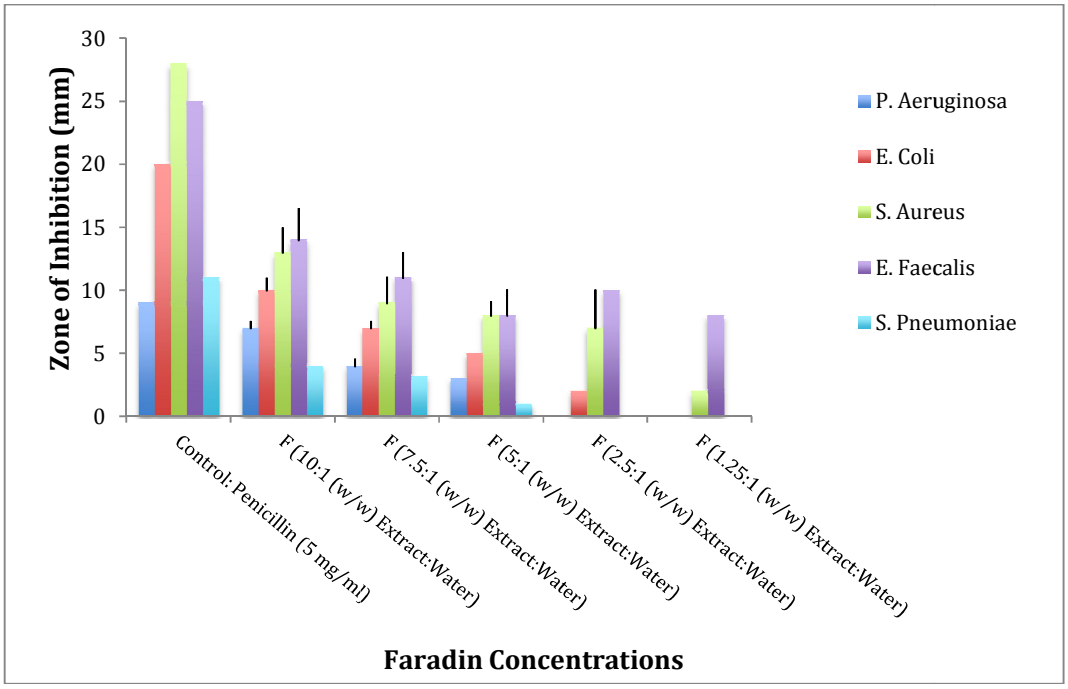


Fig. 1. Antibacterial screening of F Extract against Penicillin

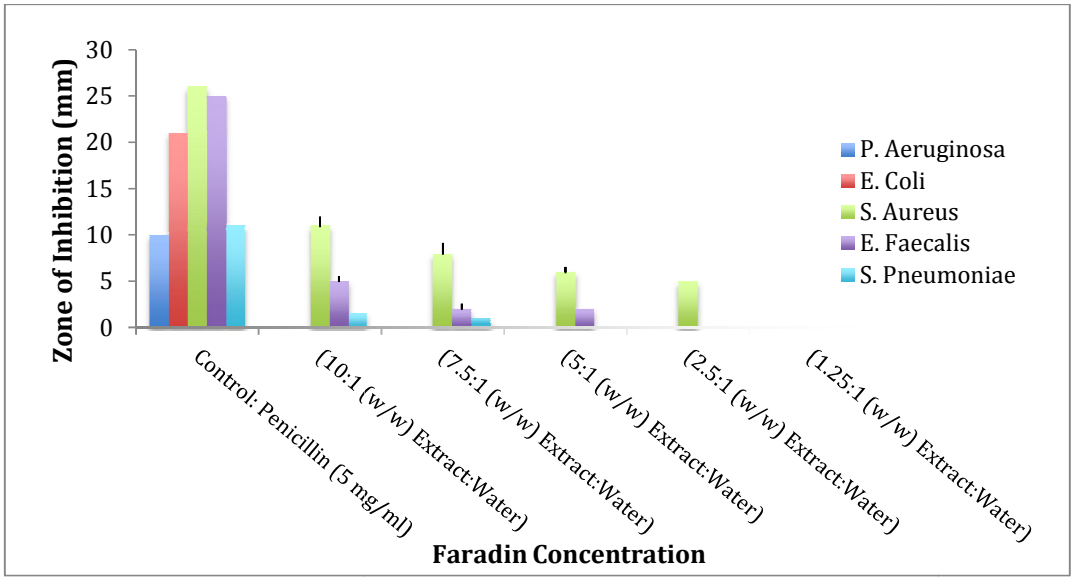


Fig. 2. Antibacterial screening of M Extract against Penicillin

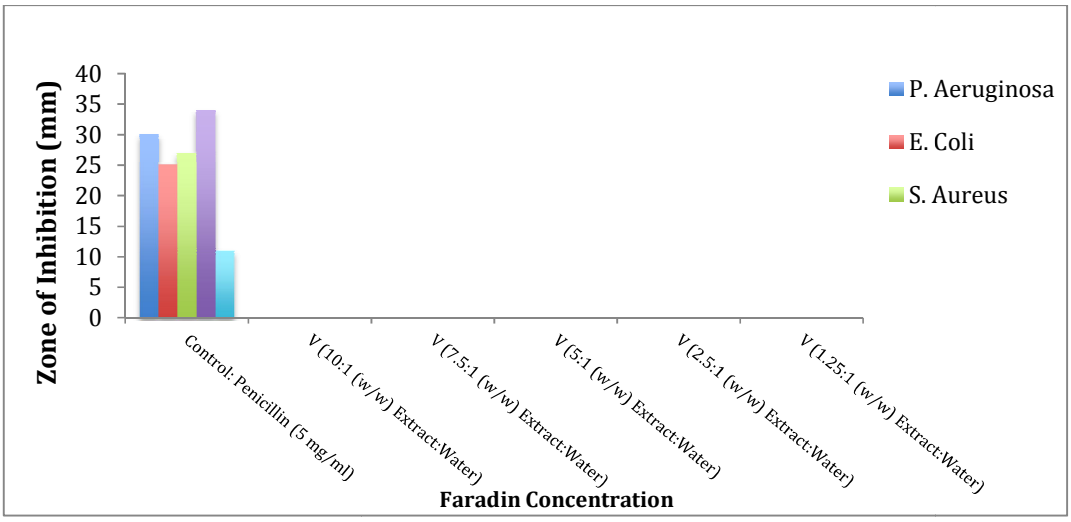


Fig. 3. Antibacterial screening of V Extract against Penicillin

The results showed the contributory antibacterial action of the single extract or lack of it to the antibacterial action of the polyherbal Faradin. Although V extract did not display antibacterial action, the phytochemicals present in it, i.e., coumarins, alkaloids and antioxidants [13] must have played a significant role either in the anti-sickling or anti-inflammatory effect. This will be investigated in the next phase of the project. In the review of Ali and Okoh, it was suggested that modulation or suppression of molecules similar to inflammasome NLRP3 mentioned earlier [2,3] could be studied in SCD using plant extracts [16]. It will be serendipity if NLR is found to be linked with co-morbid inflammation associated with SCD.

3.1.2 Anti-bacterial action of Faradin

Faradin displayed zones of inhibition towards *E. coli, P. aeruginosa, E. faecalis and S. aureus* and S. pneumonia using the same ratios as the single extracts when screened against erythromycin or penicillin. However, unlike the single extracts, the polyherbal displayed a slightly different pattern of antimicrobial sensitivity. For single extract M or F, the microorganisms *S. aureus and E. faecalis* were more susceptible. In contrast, *S. aureus and S. pneumoniae were more susceptible to the polyherbal.* Extract V showed no antibacterial action against any microorganism. A general susceptibility chart is shown in Table 1 using the antibacterial action of the CAM and single constituents versus penicillin. There was a dose-dependent susceptibility of all the bacteria species to Faradin at varied concentrations (volume ratios) that was significant ($p < 0.05$). The zones of inhibition (ZI) correspondingly ranged from 2.33 - 15.33 mm. *S. aureus* showed further susceptibility at lower concentrations range of 0.08:1 - 0.02:1 with ZI of 3.00 – 0.70 mm respectively. The ZI ranges for erythromycin and penicillin were 18-26 mm and 17- 32 mm respectively (Figs. 4 and 5).

However, the polyherbal showed more antibacterial action against gram (+) bacteria (*S. aureus, E. faecalis*, *S. pneumonia*) than Gram (-) bacteria. This is consistent with the previous reports of [17,18]. The investigators reported that Alchornea cordifolia, one of the components of Faradin had more antibacterial activity against gram (+) bacteria than Gram (-) bacteria. Within the gram (+) organisms that were used in this study, *S. aureus* was most sensitive at lower concentrations to the extract.

Gram (+) bacteria have been widely reported to be more susceptible to plant extracts compared to gram (-) bacteria [19-22]. The gram (+ve) bacteria lack an outer membrane, but are surrounded by a thick peptidoglycan mesh-like layers that could have facilitated the easy penetration of Faradin or the antibiotics into the bacterium, thus compromising the peptidoglycan layer and causing antibacterial action. On the other hand, Gram (-) bacteria have a thick outer membrane containing lipopolysaccharide and a thin peptidoglycan cell wall; therefore, this thicker outer membrane might not be permeable to the CAM or the antibiotic. The general antibacterial action could also be due to the antioxidants in Faradin that might have inhibited ROS generation, quenched free radicals, and altered

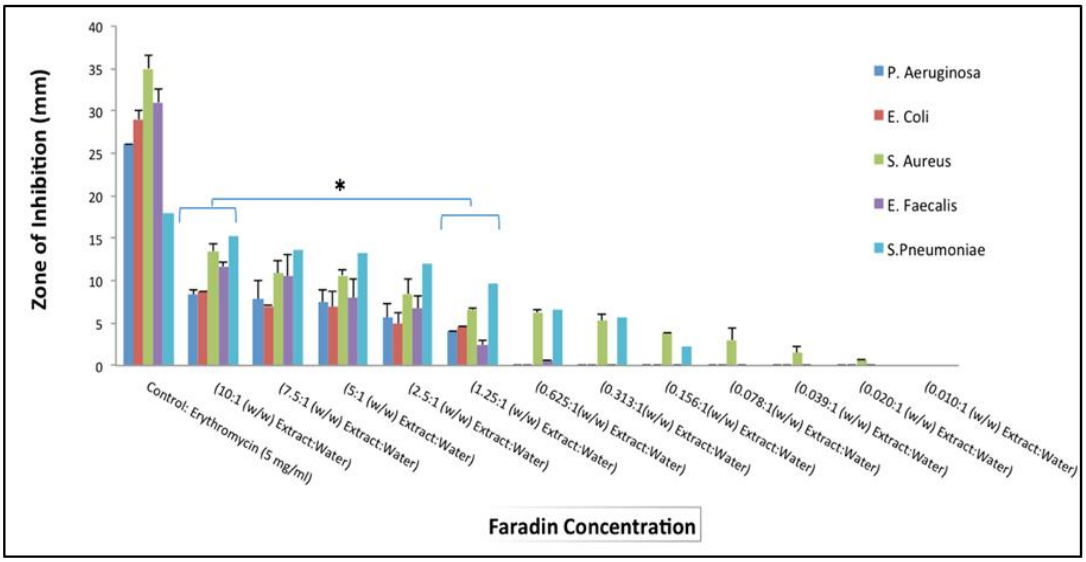


Fig. 4. Antibacterial Screening of Faradin Against Erythromycin
*(*P< 0.05)*

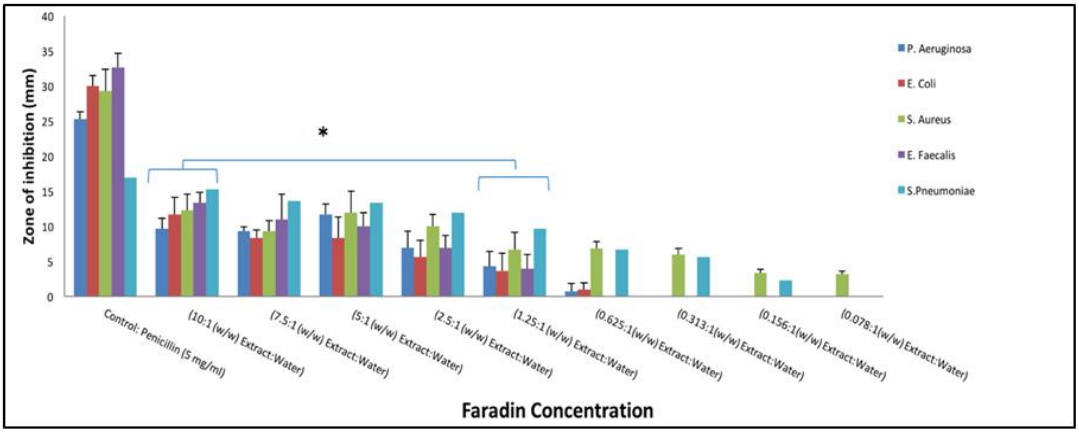


Fig. 5. Antibacterial Screening of Faradin Extract Against Penicillin
*(*P< 0.05)*

Table 1. General Antibacterial Action of Single Constituent Extract

Microorganism	F	M	V	Faradin
Gram (-)				
P. aeruginosa	++	-	-	++
E. coli	+	-	-	++
Gram (+)				
S. aureus	+++	+++	-	++++
E. faecalis	++++	++	-	++
S. pneumoniae	+	+	-	++++

(F, M or V) and Faradin Against Penicillin at Higher Concentrations
(10:1 - 1.25:1 w/w extract:water)

the intra cellular redox potential and lipid peroxidation. The result could be inhibition of microbial adherence to epithelial mucosal surfaces, and consequent selective inhibition of microbial growth [23].

3.1.3 Minimum inhibitory concentration

Faradin has an intense brown color that did not make the resazurin-based MIC color-indicative method suitable. The purple color change that could have been a signal to antibacterial effect was difficult to determine (Figs. 6A and B). As a consequent, a modified diffusion well test was used instead.

3.1.4 Modified minimum inhibitory concentration (well-diffusion method)

Due to the inability to read the color change from the from the standard MIC method (Fig. 6), the modified MIC was adopted using concentrations below the intended concentrations for the well-diffusion method. The MICs for erythromycin and penicillin were studied for each microorganism and the concentration of Faradin, calculated as mg/ml (based on the dry weight of lyophilized Faradin, excluding sugar used in the formulation as the sweetener) are shown in Tables 2A and 2B.

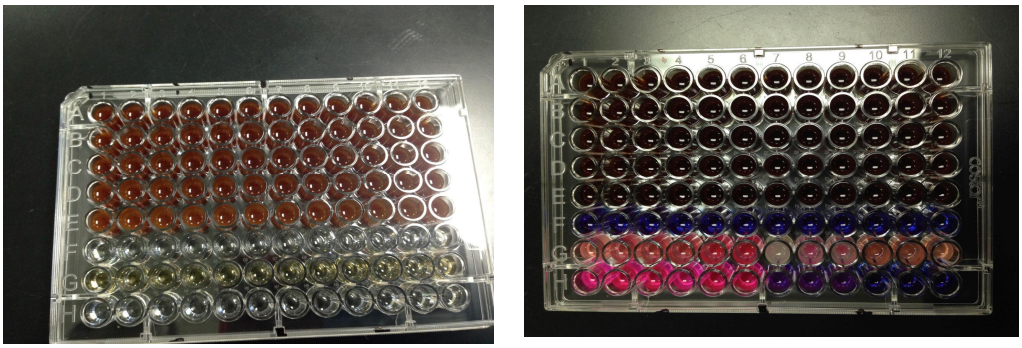
When Faradin was screened against erythromycin, the MIC for *S. aureus* was below 0.020:1 volume ratio whereas for Faradin screened against penicillin, it was 0.078:1 as discussed above (Figs. 4 and 5). The lowest MICs were observed for *S. aureus* and *S. pneumoniae*, 0.62 mg/ml and 4.84 mg/ml respectively. The MIC values for Faradin in column 1 of Tables 2A and 2B represent corresponding sensitivities of the bacteria to the polyherbal. The consistent sensitivity of S. aureus and S. pneumonia when Faradin was screened against the two antibiotics shows the specificity of Faradin in its antibacterial action. The sensitivity of the organisms to Faradin was also higher when screened against erythromycin compared to penicillin in all cases.

Considering the associated tissue damage that sometimes occur in SCD patients (as a result of tissue deoxygenation, vaso-occlusion and inflammation), there is a high potential for use of Faradin in wound healing of a damaged tissue that could have been infected with *S. aureus*. It could also be beneficial for bacterial resistant infections for which the organism is known.

3.2 Synergism of Faradin and Standard Antibiotics

3.2.1 Fractional inhibitory concentration index (FICI) for erythromycin

The calculated FICI values range was 41-5004 (Table 2A). The values are indicative of non-interaction or antagonism for the bacteria.



A B

Fig. 6. Preliminary minimum inhibitory concentration evaluation of faradin using the resazurin colorimetric method

Table 2A. MICs for Faradin and Erythromycin, and FICI Synergy Index

Bacterial strain	MIC Faradin (mg/ml)	MIC erythromycin (mg/ml)	[Faradin] mg/ml	[Erythromycin] mg/ml	FICI erythromycin	Comments (FICI)
E. coli	38.75	0.01	31	5	501.00	Non-interactive
P. aeruginosa	38.75	0.10	31	5	51.00	Non-interactive
S. aureus	0.62	0.50	31	5	41.00	Non-interactive
E. faecalis	19.38	0.001	31	5	5001.00	Non-interactive
S. pneumoniae	4.84	0.001	31	5	5004.00	Non-interactive

Table 2B. MICs for Faradin, and Penicillin, and FICI Synergy Index

Bacterial strain	MIC Faradin (mg/ml)	MIC Penicillin (mg/ml)	[Faradin] mg/ml*	[Penicillin] mg/ml	FICI Penicillin	Comments (FICI)
E. coli	19.38	1.00	31	5	5.90	Non-interactive
P. aeruginosa	19.38	0	31	5	0.88	Additive
S. aureus	2.48	0	31	5	31.00	Non-interactive
E. faecalis	38.75	0.10	31	5	51.29	Non-interactive
S. pneumoniae	4.84	0.10	31	5	53.88	Non-interactive

** [Faradin] = dry weight of lyophilized Faradin after subtracting the sugar content in the liquid Faradin formulation*

Therefore, an inference that binding of penicillin-binding proteins (PBPs) located inside the bacterial cell wall (that could cause cell lysis via interference with inhibitor of autolytic enzymes such as autolysins) may not be the responsible mechanism of reaction. Erythromycin-inhibition of bacterial protein synthesis (after entering the cell) via binding to bacterial 50S ribosomal subunit could currently also be ruled out as a mechanism of action. Otherwise, this could have resulted in interference with the translocation of amino acids during translation and assembly of proteins thus causing the antibacterial action [24].

3.2.2 Fractional inhibitory concentration index (FICI) for penicillin

The calculated FICI values range was 0.88 – 53.88 (Table 2B). The values are indicative of non-interaction or antagonism except for gram (-ve) *P. aeruginosa* that had 0.88 FICI value, indicative of an additive effect. The activity may be partly due to competition with penicillin for the penicillin-binding proteins (PBPs) in the bacterial cell wall. Synergy or additive action was ruled out for all other bacteria based on the postulated reasons stated above. The additive interaction of penicillin and Faradin observed for *P. aeruginosa* could be good clinically in co-administration. The bacteria species *is* known to be resistant to many antibiotics and it is a leading cause of nosocomial respiratory tract, urinary tract and skin infections – disease states that could be co-morbidities in SCD patients.

3.2.3 Postulated mechanism of general antibacterial activity

Aside from the explanation of possible mechanism of action of additive action of Faradin for *P. aeruginosa* explained in sections 3.1.2, the antibacterial action observed could most likely due to the antioxidants in Faradin that caused inhibition of ROS generation, quenching of free radicals, alteration of the intra cellular redox potential and lipid peroxidation. The ultimate result would be inhibition of microbial adherence to mucosal or epithelial surfaces, inhibition of endotoxin shock, and selective inhibition of microbial growth. The nature of the bacteria cell wall also contributed to the antibacterial activity with preference for gram (+) bacteria. The mechanism will be elucidated in future studies.

4. CONCLUSIONS

Polyphenols, flavonoids and antioxidants present in the M and F components of the polyherbal [11] contributed to the broader spectrum antibacterial effects. M component only showed activity against the gram (+) species - *E. faecalis, S. aureus* and *S. pneumonia.* Although the V extract has relatively low flavonoid content, the antioxidant and phenolic content contributed to the overall antibacterial activity of Faradin that showed more antibacterial action against the gram (+) species more than the single components. Due to the low MICs for *S. aureus* and *S. pneumoniae*, Faradin has high potential for management of SCD bacterial-based infection co-morbidity. In addition, the general non-synergy and non-additive action to the antibiotics could be beneficial clinically since the postulated anti-oxidation would lead to cell wall maintenance in sickle cell patients if Faradin is co-administered with the antibiotic. Determination of antibacterial mechanisms of action using methods such as lipid peroxidation and superoxide anion scavenging studies and toxicity in a whole animal model are planned for the near future.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

The study was partly funded by Roosevelt University College of Pharmacy and Elim Pediatric Pharmaceuticals Inc.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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