Research Article

Evaluation of Antimicrobial Activity of *Conyza bonariensis* Leaf Extracts against Clinically Isolated Fungi Causing Superficial Infection

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Plants have been used since long time ago to treat infectious diseases and are considered as important sources of new antimicrobial agents. In this study, crude extracts from leaves of *Conyza bonariensis* were prepared using methanol, ethyl acetate, hexane, and chloroform. Antimicrobial activity of the extracts was evaluated against pathogenic fungi causing superficial infection (*Candida albicans*, *Malassezia globosa*, and *Malassezia furfur*). Results demonstrated that all extracts had different effects against all the tested fungi with the exception of crude extract using hexane which did not show any effect against *M. furfur*. A strong effect was observed with chloroform and hexane extracts on *C. albicans* (32.60 ± 4.69 mm and 27.00 ± 1.00 mm), respectively. While, ethyl acetate and methanol extracts showed the best effect against *M. furfur* (30.80 ± 1.71 mm and 27.00 ± 1.00 mm), respectively. Moreover, the ethyl acetate showed a considerable effect on *M. globosa* (25.03 ± 1.05 mm). Minimum inhibitory concentration (MIC) of the fractions was also determined by the microbroth dilution method. The results recorded as the MIC values of the tested extracts against fungi varied from 0.19 ± 0.00 to 66.66 ± 2.86 mg/mL. Ethyl acetate was the best and powerful extract with the lowest MIC value of $0.190.19 \pm 0.00$ mg/mL for all tested fungi followed by chloroform and methanol extracts with the MIC values ranging from 0.19 ± 0.00 to 0.78 ± 0.00 mg/mL and 0.84 ± 0.68 to 1.56 mg/mL, respectively. Concerning minimum bactericidal concentration (MFC), ethyl acetate was the most potent extract with a MFC value of $0.190.19 \pm 0.00$ mg/mL for *C. albicans* and *M. furfur*. Higher (0.39 mg/mL) MFC was recorded against *M. globosa* by this extract. In conclusion, solvent extracts of some *C. bonariensis* can be used to treat infections with pathogenic fungi such as *C. albicans*, *M. furfur*, and *M. globosa*. Further studies should consider this plant as one of the best candidates for the discovery of potent antimicrobial compounds that treat superficial infections.

1. Introduction

According to the WHO [1], traditional medicine is defined as health practice, approach, knowledge, and beliefs incorporating plant, animal, and mineral-based medicines, spiritual therapies, manual techniques, and exercises applied singularly or in combination to treat, diagnose, and prevent illness or maintain wellbeing. Traditional medicine and medicinal plants have been employed by the majority of the world population for thousands of years. According to fossil records, the human use of plants as medicines may be drawn back at least 60,000 years [2]. Until the establishment of the 19th century, all medicines were traditional. Yet, in several developing countries, it is true that for the majority of rural population, traditional medicine is the only primary medicine for healthcare [3]. Medicinal plants have provided good sources of antimicrobial agents against several infectious pathogens including bacteria and fungi [4].

Either due to limited availability or affordability of pharmaceutical medicines, about 80% of the rural

population in sub-Saharan Africa depends on traditional herbal therapies for primary healthcare [1]. Similarly, in Ethiopia, 80% of the population depends on the use of traditional medicine [5]. Furthermore, Ethiopia is one of the six countries of the world where about 60% of plants with healing potential are said to be indigenous [6]. The country is believed to be a home for approximately 6,500 species of higher plants with about 12% endemic, hence making it one of the six plant biodiversity-rich countries of Africa [7, 8], and the country has a long history of using medicinal plants to treat a variety of human ailments [9].

Ethiopia is with a wealth of unexplored natural products, and it is an ideal place to search for new medicine for the treatment of infectious diseases. Eventhough some studies have been conducted to explore the antimicrobial potential of various medicinal plants, these studies are insufficient as compared to the current widespread problem and prevalence of drug resistance microbe as well as the urgent needs of new medicine [10, 11].

The skin being the outermost and first line of protection is simply exposed to physical agents and different pathogens leading to several infections [12]. Fungal pathogens commonly dermatophytes or superficial mycotic fungi of humans and animals not only infect the keratinized tissues of the skin but also infect nails and hairs [13]. They are most likely found in hot humid areas. These fungi can easily digest the keratinized tissue by releasing sulphite, exoprotease, and endoprotease [13]. Sulphite being a reducing agent broke the disulphide bonds of keratin protein and made them more vulnerable to fungal proteases enzymes. Human infections, particularly those involving the skin and mucosal surface constitute a serious problem, especially in tropical and subtropical developing countries, dermatophytes and *Candida* sp., being the most frequent pathogens.

C. albicans is an opportunistic fungal pathogen of humans. Although a normal part of our gastrointestinal flora, vagina, and mucosal cavity, *C. albicans* has the ability to colonize nearly every human tissue and organ, causing serious, invasive infections [14]. *C. albicans* is a normal resident of the human gastrointestinal tract; it is also the most common fungal pathogen of humans, causing both mucosal and systemic infections, particularly in immunocompromised or whose natural flora has been altered patients [15]. *Candida* species are yeast-type fungi. *C. albicans* is the most common pathogen among the *Candida* species [16]. Lesions caused by *C. albicans* appear as white patches on the skin or mucus membrane, hence the name *C. albicans*. Other species within this genus that cause disease include *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* [17]. Phytochemical extracts from medicinal plants have been used to treat infectious diseases such as candidiasis in developing world [18]. Study reports from Pramila et al.[19] and Hussein et al. [20] showed that herbal extracts are generally effective against *C. albicans*.

Tinea versicolor also known as pityriasis versicolor is a condition characterized by a skin eruption on the trunk and proximal extremities. A yeast-like fungus called *M. globosa* is the major cause of tinea versicolor. *M. furfur* is another causative agent for this disease [21, 22]. Both yeasts are normally found on the human skin and become troublesome only under certain circumstances, such as a warm and humid environment [21, 23].

Dandruff is a skin condition that mainly affects the scalp. Symptoms include flaking and itchy scalp [24] and inflammation of the skin (seborrhoeic dermatitis) [25]. Red and greasy patches of the skin and a tingly feeling on the skin are also symptoms. The responsible microorganism that affects the scalp is a fungus, *M. furfur* [26]. This disease is also caused by other fungi, namely, *M. globosa* [27] and *M. restricta* [28]. *M. globosa* metabolizes triglycerides present in sebum by the expression of lipase, resulting in a lipid byproduct oleic acid [27].

Natural products such as extracts and essential oils from native plants have been important sources of products for the developing countries in treating common infections including fungal diseases. Some studies have demonstrated that the plant extract has been used traditionally to treat a number of infectious diseases caused by bacteria and fungi [13].

The species of *Conyza* have a great capacity of adaptability, which allows them to occur in different soil and climatic conditions [29]. *C. bonariensis*, which is an annual or short-lived perennial weed of the Asteraceae family [30], is widely used as a folk medicine in the treatment of rheumatism, gout, cystitis, nephritis, dysmenorrhea, tooth pain, and headache; it is also reported to have an anti-ulcerogenic and anticoagulant activity [31]. *C. bonariensis* is also used to treat a variety of skin conditions [32].

Due to the availability of rich biodiversity and highly unexplored natural resources in Ethiopia, focusing on searching for novel, highly effective, better active, safe, and affordable antimicrobial agents must be prior activity. Screening using in vitro evaluation is a useful tool for the discovery of new potential antifungal agents from natural products such as essential oils and extracts derived from plants. Therefore, the current study is aimed at evaluating the antimicrobial potential of *C. bonariensis* leaf extract against selected human pathogenic fungi causing superficial infection.

2. Materials and Methods

2.1. Study Area and Period. The current study was conducted at Tewodros Campus, Microbiology Laboratory, Institute of Biotechnology, University of Gondar, Gondar town, Northwest Ethiopia. Gondar town is located 738 Km away from Addis Ababa, capital city of Ethiopia. The town has 12°C 36′north latitude and 37°C 28′ east longitude with an elevation of 2133 meter above sea level. Average maximum and minimum temperature is 29°C (in March and May) and 10°C (in January and December), respectively. The mean relative humidity for an average year is recorded as 55.7%, and on monthly basis, it ranges from 40% in January to 79% in July. According to a report by CSA [33], Gondar has a population of 299,969.

2.2. Study Design. An in vitro experimental laboratory-based study was conducted from January to September 2019 to evaluate antimicrobial activity of leaf extracts of the plant *C. bonariensis* against available clinical fungal pathogens.

2.3. Plant Material Collection and Identification. Leaves of *C. bonariensis* plant were collected from Gondar City in University of Gondar compound. Normally, non-contaminated and nondiseased leaves were used for the study (Figure 1). The plant leaves were selected based on the information given by local traditional healers (practitioners) in the area, Gondar town. Taxonomical identification and verification were performed at Department of Biology by Mr. Abiyou Eniyew, a Botanist, College of Natural and Computational Sciences, University of Gondar. Last, voucher of the specimen with deposition number 001/YGL/2019 was deposited in the College of Natural and Computational Sciences Herbarium, University of Gondar.

2.4. Preparation of Plant Crude Extracts. Leaves of the plant were washed with tap water and air dried under shade at room temperature for two weeks. Then, it was chopped, powdered, and kept in an air tight container until needed for the extraction process. Then, the powder was extracted by the maceration technique with four solvents as follows: methanol (80%), chloroform (99.5%), ethyl acetate (99%), and n-hexane (99%). Ground and powdered plant materials were soaked with each solvent separately at 10 : 1 solvent-to sample ratio (v/w) [34–36].

A 120 g of powdered plant material was obtained, and 30 g was used for each solvent. Thus, 30 g powder was macerated in 300 mL of each solvent in extraction bottles, such that the level of the solvent was above that of the plant material. The macerated mixtures were then left on the shaker for 72 h at room temperature.

Each extract was filtered through Whatman No.1 filter paper and concentrated with a Buchi Rota vapor R-200 and then transferred to appropriately labeled vials and allowed to stand at room temperature to permit evaporation of residual solvents. Methanol extract was concentrated using a ly-ophilizer to remove water residue. After all, 5, 5, 5, and 4 g of crude methanolic, chloroform, ethyl acetate, and n-hexane extracts were obtained, respectively. Then, the extracts were stored under refrigeration at 4°C for further studies.

2.5. Qualitative Analysis of Phytochemicals. The presence of different phytochemical constituents of *C. bonariensis* was detected using standard procedures. The presence of phe-nolic compounds was detected using the protocol of Martin et al. [37]. Flavonoids, terpenoids, cardiac glycosides, al-kaloids, saponins, and tannins were characterized using the method of Ayoola et al. [38]. Steroids [39] and anthraqui-nones [40] were also detected.

2.6. Screening for Antifungal Activity. Antifungal activities of different extracts were determined using the agar well dif-fusion assay [41]. The test pathogenic fungi (*C. albicans*, *M. globosa*, and *M. furfur*) were obtained from the Uni-versity of Gondar Comprehensive Specialized Hospital. These pathogenic fungi were first grown on Sabouraud dextrose broth, incubated for 3 days at 29°C. Fresh fungal cultures were spread on the surface of Sabouraud dextrose

FIGURE 1: Image of *C. bonariensis* taken by Tamene Milkessa Jiru.

agar plates and incubated for 3 days at 29°C. Wells were made in Sabouraud dextrose agar with the help of sterile cork borer keeping enough distance between them. About 100 mg/mL stock of each crude extract was prepared by dissolving 100 mg of dried plant powder in 1 mL of 10% DMSO and added to the wells, while one well was sup-plemented with DMSO, which served as the negative con-trol, and ketoconazole as the positive controls [42].The zones of inhibition were then measured after 72 h of the incubation period. All the experiments were conducted in triplicates.

2.7. Determination of MIC. Determination of MIC of each crude extract was performed using the broth microdilution method [43]. First, 100 μL of Sabouraud dextrose broth was added to all the wells of a 96-well microtiter plate. Then, about 100 μL of each dissolved crude extract (100 mg/mL) was added in the first well and serially diluted row by row to give 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, and 0.19 mg/mL, and 100 μL of the mixture was discarded from the last row, thus leaving each diluted well with a volume of 100 μL, except for the positive and negative controls. The inocula of test strains prepared from fresh overnight cultures were adjusted to 0.5 McFarland standard, and 100 μL was poured to the wells of a 96-well microtiter plate. Wells containing microbial sus-pensions, growth medium, as well as DMSO were used as negative control. Wells containing microbial suspensions, growth medium, and ketoconazole were used as positive control.

The microtiter plates were incubated at 29°C for 72 h for fungi, and then, 20 μL of resazurin was added to each well to indicate respiratory activity, and a change in color from blue to pink would be determined after incubating it at 29°C for 24 h. The MICs were the lowest concentration where no viability was observed after 24 h on the basis of metabolic activity. All the growth formation below the MIC or the growth of one or two colonies or film of growth will be discarded or disregarded [44, 45].

2.8. Determination of MFC. Minimum MFC is the lowest extract concentration that completely exterminates the microbial population. Microbial cells from the MIC test plates were subcultured on the fresh Sabouraud dextrose

agar media for fungi. The plates were then incubated at 29°C for 3 days. Plates that did not show growth of the colony or 99.9% reduction of CFU on the solid agar medium after subculturing were considered to be the MFC [46, 47]. The experiments were carried out in triplicates.

2.9. Data Analysis and Interpretation. The data collected was entered to Microsoft Excel 2013 and transferred to SPSS version 20 for analysis. The experimental results were expressed as mean ± standard deviation (SD) of three replicates. Where applicable, the data were subjected to one-way analysis of variance (ANOVA), and differences between samples were determined by the two-tailed *t*-test after Bonferroni error correction of the predictive value. *P* values less than 0.05 will be considered statistically significant.

3. Results

3.1. Qualitative Analysis of Phytochemicals. In the current study, chloroform, ethyl acetate, hexane, and methanol extracts of the plant *C. bonariensis* were tested qualitatively for the presence of phytochemicals. All the plant extracts were found to contain steroids, terpenoids, phenols, saponins, tannins, alkaloids, anthraquinones, flavonoids, and glycosides.

3.2. Antifungal Activities. The result of agar well diffusion assay demonstrated that all extracts had different effects against all the tested fungi with the exception of crude extract using hexane which did not show any effect against *M. furfur*. A strong effect was observed with chloroform and hexane extracts on *C. albicans* (32.60 ± 4.69 mm and 27.00 ± 1.00 mm), respectively. While, the ethyl acetate and methanol extract showed the best effect 30.80 ± 1.71 mm and 27.00 ± 1.00 mm, respectively, against *M. furfur*. Moreover, the ethyl acetate showed a considerable effect on *M. globosa* (25.03 ± 1.05 mm) (Table 1).

The comparison of the effect different extracts against each fungal pathogen was evaluated in the agar well diffusion assay. According to the result, even if the *C. albicans* showed a variable degree of susceptibility to all extracts, the best result was recorded by chloroform extract (32.60 ± 4.69 mm), followed by ethyl acetate (30.10 ± 6.18 mm). But the effects observed by both extracts were lower compared to ketoconazole (44.40 ± 1.69 mm).

On the other hand, *M. furfur* was highly susceptible to ethyl acetate crude extract followed by methanol extract. These effects were more preferable, since there was no any effect produced by ketoconazole against *M. furfur*. Furthermore, *M. globosa* was sensitive to all extracts with variable degrees. The best effect was observed by ethyl acetate (25.03 ± 1.05 mm) followed by methanol (21.33 ± 1.53 mm). In addition, the effects made by all extracts were still better compared to the control ketoconazole (11.00 ± 0.00 mm) (Table 1). No effect was exhibited by DMSO against each fungal pathogen that causes superficial infection.

Statistically, no significant difference result was seen against *C. albicans* between chloroform, ethyl acetate, and hexane extracts ($p < 0.05$), but there was a significant difference between those extracts and methanol extract as well as both positive and negative controls ($p < 0.05$). Concerning *M. furfur,* statistically significant higher result was seen by ethyl acetate, methanol, and chloroform extracts, compared to ketoconazole and DMSO ($p < 0.05$).

The result of data analysis indicated that there was no significant antimicrobial effect difference between ethyl acetate and methanol extracts against *M. globosa*; however, their effect was significantly different from ketoconazole ($p < 0.05$) (Table 1). There was also no significance between extracts of chloroform and hexane.

3.3. Determination of MIC. The results recorded as the MIC values of the tested extracts against fungi varied from 0.19 ± 0.00 to 66.66 ± 2.86 mg/mL. Ethyl acetate was the best and powerful extract with the lowest MIC value of 0.19 ± 0.00 mg/mL for all tested fungi followed by chloroform and methanol extracts with the MIC values ranging (0.19 ± 0.00 to 0.78 ± 0.00 mg/mL) and (0.84 ± 0.68 to 1.56 ± 0.00 mg/mL), respectively. On the contrary, the poor MIC value was recorded by hexane extract from 1.56 ± 0.00 to 66.66 ± 2.86 mg/mL (Table 2).

3.4. Determination of MFC. Based on the result obtained, the MFC values of the tested extracts generally varied in the range from 0.19 ± 0.00 to 83.33 ± 1.83 mg/mL. Ethyl acetate was the most potent extract with a MFC value of 0.19 mg/mL for *C. albicans* and *M. furfur*. Higher (0.39 mg/mL) MFC was recorded against *M. globosa* by this extract. The lowest and highest MFC values recorded by chloroform and methanol extracts were (0.19 ± 0.00 and 0.78 ± 0.00 mg/mL; 0.78 ± 0.00 and 3.12 ± 0.00 mg/mL) on *C. albicans* and *M. globosa*, respectively. Very high MFC value (83.33 ± 1.83 mg/mL) was observed on *M. furfur* by hexane extract.

All extracts showed considerable effects on *C. albicans*. But the best was recorded by chloroform and ethyl acetate extracts at similar concentration (0.19 ± 0.00 mg/mL). On the other hand, *M. furfur* and *M. globosa* could be killed by ethyl acetate extract at the concentrations of 0.19 ± 0.00 mg/mL and 0.39 ± 0.00 mg/mL, respectively (Table 3).

4. Discussion

Medicinal plants are abundant sources of antimicrobial molecules; for this reason, a wide range of their extracts are used to treat several infections [48], and numerous works have been performed to examine the antimicrobial effects of the extracts from roots, stem, leaves, flowers, or seeds [32, 49, 50], and the beneficial medicinal effects of plant materials typically result from the combination of secondary products present in plants. Many studies conducted in different parts of the world showed that leaves are used more than others parts of a plant [32, 51–54].

In this study, prior to testing antibacterial activities, crude extracts of *C. bonariensis* leaves were qualitatively tested for the presence of phytochemicals. All extracts were found to contain steroids, terpenoids, phenols, saponins,

TABLE 1: Antifungal activities of different extracts determined using the agar well diffusion assay.

Crude extracts	C. albicans	M. furfur	M. globosa
Chloroform extract	32.60 ± 4.69^b	20.63 ± 0.65^b	12.33 ± 0.58^b
Ethyl acetate extract	30.10 ± 6.18^b	30.80 ± 1.71^a	25.03 ± 1.05^a
Hexane extract	27.00 ± 1.00^b	0.00 ± 0.00^c	12.67 ± 0.58^b
Methanol extract	16.57 ± 1.40^c	27.00 ± 1.00^a	21.33 ± 1.53^a
KC	44.40 ± 1.69^a	0.00 ± 0.00^c	11.00 ± 0.00^b
DMSO	0.00 ± 0.00^d	0.00 ± 0.00^c	0.00 ± 0.00^c

The values of average zones of inhibitions are expressed as mean ± SD ($n = 3$) within each column; means are significantly different ($p < 0.05$), unless they have a common letter. DMSO, dimethyl sulphoxide; KC, ketoconazole.

TABLE 2: MIC (mg/mL) value of each extract against fungi.

Crude extracts	C. albicans	M. furfur	M. globosa
Chloroform extract	0.19 ± 0.00	0.52 ± 0.22	0.78 ± 0.00
Ethyl acetate extract	0.19 ± 0.00	0.19 ± 0.0	0.19 ± 0.00
Hexane extract	0.52 ± 0.22	66.66 ± 2.86	1.56 ± 0.00
Methanol extract	0.84 ± 0.68	0.65 ± 0.22	1.56 ± 0.00

TABLE 3: MFC (mg/mL) value of each extract against skin infecting fungi.

Crude extracts	C. albicans	M. furfur	M. globosa
Chloroform extract	0.19 ± 0.00	0.78 ± 0.00	1.56 ± 0.00
Ethyl acetate extract	0.19 ± 0.00	0.19 ± 0.00	0.39 ± 0.00
Hexane extract	0.52 ± 0.22	83.33 ± 1.83	3.12 ± 0.00
Methanol extract	0.78 ± 0.00	1.56 ± 0.00	3.12 ± 0.00

Average MFC values are expressed as mean ± SD ($n = 3$); analysis was performed with one-way ANOVA followed by the Bonferroni test.

tannins, alkaloids, anthraquinones, flavonoids, and glycoside, which means that the studied plant includes all these compounds that had contributed to its antimicrobial activity against the tested fungal pathogens. Secondary metabolites belonging to different phytochemicals have been reported from members of genus *Conyza* including alkaloids, volatile oils, terpenoids, phenols, flavonoids, and hydrolysable tannins [55].

In the current study, solvent extracts of leaves of *C. bonariensis* were used. The antimicrobial activity of chloroform, ethyl acetate, hexane, and methanol extracts from the plant *C. bonariensis* leaves were tested against three fungal pathogens that cause superficial infections. It was found that the plant had antimicrobial activity against those microbes. This finding was in line with the studies conducted by Shah et al. [56], Musembei and Joyce [57], and Mussin et al. [32]. The possible explanation for this could be the plant *C. bonariensis* is rich in phytochemical compounds with potential antimicrobial activities as confirmed from experiment.

In this study, all the crude extracts were applied against different fungal pathogens. Most of them showed different antimicrobial activities to the tested organisms. The possible reason for this different antimicrobial activity could be the susceptibility pattern of the pathogens and the differences in

mechanism of actions of the metabolites in each solvent fraction, the method of extraction used, the different geographical and environmental conditions during the growth of the plant, and the age of the plant.

The current study revealed that ethyl acetate and hexane extract showed maximum and minimum average zones of inhibition, respectively. This result was in line with the study conducted by Shah et al. [56] at Pakistan who obtained similar results. The possible justification ethyl acetate could be the best solvent to extract active ingredients, which powerfully combat the tested microbes.

The average zones of inhibition formed by ethyl acetate, chloroform, methanol, and hexane extracts against *C. albicans* were 30.10 ± 6.18 mm, 32.60 ± 4.69 mm, 16.57 ± 1.40 mm, and 27.00 ± 1.00 mm, respectively. The finding of this study is a bit lower than Shah et al. [56] who obtained a higher inhibition effect of ethyl acetate, chloroform, and methanol extract with inhibitory zone of 35 mm, 28 mm, and 25 mm, respectively. Again, another better result was also reported by Shah et al. [58] from Pakistan on related species of plant with the maximum effect against *C. albicans* produced by chloroform, n-hexane, and ethyl acetate fraction with inhibitory zone of 45 mm, 40 mm, and 33 mm, respectively.

DMSO which was used as a control showed no effect against the growth each pathogenic fungi causing superficial infection. All pathogenic fungi studied grew in the absence of extract.

The results recorded as the MIC values of the tested extracts against *C. albicans* varied from 0.19 to 0.84 mg/mL. According to the study conducted by Ahmed et al. [4] in Egypt on *C. bonariensis*, a MIC value of >800 mg/mL against *C. albicans* was found which was poor compared to the present finding. This could be the difference in the methodology utilized. Similarly, the MFC values of the tested extracts against the three pathogenic fungi causing supercritical infections generally varied in the range from 0.19 ± 0.00 to $83.33 \pm \pm 1.83$ mg/mL.

5. Conclusion

This study investigated the antimicrobial activities of the plant *C. bonariensis*. The plant extracts showed antimicrobial activity when used against fungal pathogens. Results indicated that the ethyl acetate extract demonstrated significant antifungal activities against the growth of pathogenic fungi, but n-hexane showed less antibacterial activity against any of the test microbes relatively. This result can pave the way towards the move for the discovery of new efficacious, less toxic, and inexpensive plant-based medicines used for controlling pathogenic fungi causing superficial infections. Moreover, it might give the answer to the currently existing and newly emerging fear of multidrug resistance pathogenic microorganisms.

Data Availability

The data used to support the findings of this study are included within the article.

Disclosure

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

This work was carried out under the supervision of TMJ. TMJ also helped in editing the manuscript. Both authors read and approved the final manuscript.

Acknowledgments

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