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Antidepressant activity of Methanolic Extracts of *Areca catechu Nuts* in Mice

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The Pharmaceutical Society of Kenya (PSK) is a representative organization that was formed enabling Pharmacists' to employ their professional expertise in the care of patients.

Established in 1964, PSK has its roots in the Pharmaceutical Society of East Africa, which was registered in 1950. Since its formation, PSK continues to promote a common standard for professional conduct and code of ethics for its members, as well as advocate for the welfare of Pharmacists.

EDITORIAL

Plant Secondary Metabolites as Suitable Treatment Option to Overcome Drug Resistance

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Emergence of resistant bacterial strains represents a major threat to public health care. In addition, the rising cases of cancer and depression and their resistance to the currently available drugs worldwide calls for alternative treatment options. Plants and herbs present the most efficient and safe source of medication. Compared to their synthetic counter-parts, bioactive compounds of plant origin are cheaper, more readily available and have fewer side effects. According to World Health Organization (WHO), 80% of developing countries use conventional medicines derived from plants, with above 100 countries having developed regulations for medicinal plants.

Due to their immobile nature, plants are continually exposed to numerous biotic and abiotic stresses, such as pathogen attack, UV-irradiation, nutritional imbalances, salinity and extreme temperatures in their natural environments. To protect themselves against pathogen invasion, oxidative stress and to deter herbivores, they produce multifunctional secondary metabolites such as phenolic compounds, terpenes, sulfur and nitrogen containing metabolites. Phenolic compounds are the most widely distributed secondary metabolites and present a diverse group of compounds that include simple phenols, flavonoids, tannins, catechol melanins and lignins. Accumulation of the different secondary metabolites is highly dependent on environmental factors. For example, upregulation of genes in the phenylpropanoid pathway and the accumulation of phenolics in response to environmental stress has been reported.

Plant secondary metabolites are the sources of bioactive substances for drugs of plant origin. They have been reported to have activity to treat many diseases including infections, cancers and depression. Antimicrobial compounds from medicinal plants use a variety of mechanisms to inhibit growth of viruses, bacteria, fungi and protozoa. These include bacterial cell disruption which leads to increased cell permeability and leakage of constituents; pH disturbance; intracytoplasmic damage; inhibition of protein synthesis; and DNA damage. A combination of these mechanisms is of great value in overcoming the burden of resistant microbes. The metabolites exert anticancer activity by targeting signaling and various metabolic pathways, thus controlling angiogenesis; inhibit microtubule assembly formation in cells, leading to apoptosis.

Of the prescribed anticancer drugs, 60% are derivatives of plant metabolites. Gallic acid, a natural antioxidant and a polyhydroxy phenolic compound, that is present in a variety of plants, has been shown to induce apoptosis in lung cancer, colon adenocarcinoma cell lines and leukemia. Tannic acid (another natural antioxidant which is a mixture of digallic acid esters of glucose) anticancer activity is attributed to its ability to inhibit carcinogens activation; arrest cell cycle; reduce rate of cell proliferation, cell migration, and adhesion of several cancer cell lines; and to induce apoptosis. Alkaloids exhibit anticancer properties by restraining topoisomerase (an enzyme that is associated with the replication of DNA), initiating apoptosis, and modulating various other intracellular targets and signaling pathway.

An increased use of medicinal plants and herbs worldwide for developing nutraceuticals for treatment of depression, anxiety and other psychiatric disorders has been reported. Plant extracts have been shown to affect the nervous system and exert antidepressant effects through synaptic regulation of serotonin, noradrenaline and dopamine; and inflammatory mediators. Lavender, lemon balm hops, valerian and maypop plants and their products, for examples, have been shown in clinical trials to relieve mild forms of neurological disorders such as, especially, anxiety, depression and stress.

Despite plant secondary metabolites having promising therapeutic properties including antimicrobial, anticancer and antidepressive activities, their clinical use is still limited due to their physico-chemical properties such as limited bio-availability and/or their toxicity. There is therefore great need to come up with innovative ways to improve bioavailabity and reduce cytotoxic effects. This may include identifying and purifying the active elements, modifying the chemical structure of promising compounds and/or packaging of the plant extracts into nano-formulations. This however, calls for the use of modern analytical procedures.

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Evaluation of antimicrobial activity of biogenic synthesized silver nanoparticles using *Artocarpus heterophyllus* lam peel and fiber extract

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Abstract

The growing threat of antimicrobial resistance (AMR) necessitates a race toward the discovery of new and more effective antibiotics as well as the protection of existing drugs. A viable alternative to the traditional chemical synthesis of antibiotics is the biogenic synthesis of nanoparticles utilizing plants. This study evaluates the antimicrobial properties of biogenically synthesized silver nanoparticles using *Artocarpus heterophyllus* (common name - jackfruit) and the plant here after will be referred to using its common name, jackfruit. Silver nanoparticles were synthesized using the powdered jackfruit peel (AgNpP) and fiber plant extract (AgNpF) and silver nitrate. Characterization of the synthesized nanoparticles was done using UV-VIS, FTRIR spectral, and Voltammetric analysis. The antimicrobial activity of the synthesized silver nanoparticles against *Staphylococcus aureus* and *Escherichia coli* was also evaluated. The UV-VIS spectral showed a peak between 390-440nm confirming the presence of silver nanoparticles. FTIR confirmed the presence of silver nanoparticles since two major peaks were formed at 3300cm^{-1} and 1600cm^{-1} for both the extracts. Voltammetry showed a redox reaction taking place indicating that silver nitrate was reduced, and silver nanoparticles were being formed. The synthesized nanoparticles showed activity against *S.aureus* and *E.coli*. The peel and fiber nanoparticles confirmed antibacterial effect and when combined with antibiotics, it confirmed synergism with *Staphylococcus aureus* bacteria and antagonism on *Escherichia coli* bacteria. A combination of amoxicillin with AgNpPs and AgNpFs showed different antibacterial activity against E.coli of 18mm and 14.67mm respectively, whereas, on S.aureus, the zone of inhibition was similar at 30.00mm and 30.33mm. On the other hand, a combination of flucloxacillin with AgNpPs and AgNpFs showed different antibacterial activity against *E. coli* at 12.67mm and 16mm respectively, whereas, on *S. aureus*, the zone of inhibition was similar at 11.67mm and 19.67mm. The zone of inhibition of independent amoxicillin, flucloxacillin, AgNpPs, and AgNpFs on *S. aureus* was 25.67mm, 17.33mm, 16mm, and 13.67mm, respectively whereas on *E.coli* were 21mm, 19.33mm, 11.67mm and 21.67mm respectively. The silver nanoparticles from the waste fruit peels and waste fruit fibers of jackfruit were synthesized and characterized. The antibacterial activity as well as the synergistic and antagonistic activity were achieved.

Key words: *Antimicrobial resistance; Nanotechnology; Artocarpus heterophyllus (Jackfruit); Silver nanoparticles; Antimicrobial activity.*

Introduction

Over the years, the demand for antibiotics in the health care system has increased globally by 70% and between 2000 and 2016, the daily doses increased from 21.1 billion to 34.8 billion and continue to increase over the years claiming more than 800,000 annually (1). According to reports from the World Health Organization (WHO), 82% of the population from Asia and Africa still depend on traditional herbal medicines compared to conventional medications (2). However, most of these herbal remedies are uncharacterized and their efficacy has not been authenticated (2).

Antimicrobial resistance (AMR) is the ability of microorganisms such as bacteria, viruses, or parasites to prevent antimicrobial agents like antibiotics, antivirals, and antimalarials to affect them (2). The growing threat of AMR necessitates a race towards the discovery of new and more effective antibiotics as well as the protection of existing drugs and that is why nanotechnology is considered (3).

Nanotechnology is one of the emerging trends that is growing rapidly in research in the modern era of material science. It involves the use of particles with a size of 1-100nm which gives a high surface area to volume ratio. Such nanoparticles have been shown to exhibit certain properties of medical importance due to their size, morphology, shape, origin, or even composition (4). Silver is a very strong antibacterial and is highly toxic to cells where it can cause bacterial cell wall damage, it can inhibit bacterial cell growth and/or affect metabolism in a cell since the silver ions can disrupt either proteins or nucleic acid in the cells. In addition, it can also affect protein synthesis or permeability of the cell membrane resulting in the death of the cell. Furthermore, it can generate reactive oxygen species and therefore contribute to killing the microbes (3). Silver nanoparticles (AgNPs) exert these mechanisms in parallel making them very good antibacterial agents. In addition to this, silver nanoparticles can also be used against fungi as well as show effectiveness against efflux pumps and biofilms formed by bacteria. This means that it can be used to overcome the

microbial resistance resulting from conventional antibiotics. Synthesis of AgNPs is mainly through chemical reduction and this is a common method due to it being economical and easier to use. Chemical reduction occurs after conversion of silver to silver ions using reducing agents such as sodium borohydride or sodium citrate (3). However, this method has a major drawback since it results in the adsorption of toxic materials on the material's surface, therefore environmentally friendly methods can be used. It is important to consider alternative safe or greener methods for the synthesis of AgNPs while maintaining the bactericidal properties and reducing the impact of the production process on the environment (3).

Synthesis of nanoparticles using biological methods is considered advantageous since it slows down the kinetics and offers better manipulation control over the growth and stabilization of crystals (4). Generally, biogenic synthesis is more advantageous than the routine chemical or physical methods since it is cost-effective, eco-environmental, scaling up is easy and there is no requirement of high pressures, high temperature, or high energy (4). In addition to this, the AgNPs can combine with active components in plants and this means that it has better biological effects compared to those synthesized chemically (5). It is thought that when AgNPs combine with certain antibiotics, they can have either a synergistic effect or an antagonistic effect. The effect it has is based on the chemical interaction between the components (5). For this study, the waste peels, and fibers of Jackfruit were used to synthesize silver nanoparticles. Research done previously has shown that both the peel and the fiber from jackfruit have some antimicrobial properties (6) and combing this with silver nanoparticles could enhance the activity by two folds since silver nanoparticles have bactericidal activity as well as the jackfruit extracts. The main components present in the peel of Jackfruit includes cellulose, starch, pectin, protein, crude fiber, total sugars, moisture content and ash content. The majority of component occupied is the polyphenols and this occupies about a third of the total composition. The polyphenols present in the peels of jackfruit acts both as a reducing and stabilizing agent in the synthesis of silver nanoparticles (6). This work, therefore, evaluated the antimicrobial properties of biogenic synthesized silver nanoparticles (AgNPs) using Jackfruit.

Methodology

Materials

Jackfruits were bought from Sabasaba market in Murang'a county and from the city market in Nairobi and transported to the USIU-Africa's botany laboratory where they were assessed by the University's botanist and taxonomist to ensure that the fruit obtained is Jackfruit. Six jackfruits each weighing between 6.6-6.8 kilograms were used. The reagents and chemicals used including analytical grade silver nitrate, trisodium citrate solution of 1%, and sodium hydroxide were sourced from Sigma-Aldrich (Germany).

Other materials required were antibiotics such as amoxicillin, flucloxacillin, phosphate buffer (pH 7.2), Mueller-Hinton agar, *Staphylococcus aureus* and *Escherichia coli*.

Preparation of jackfruit peel and fiber extracts

The jackfruits were peeled using a sharp knife to remove the peels and the fiber. Both the peel and fiber were placed in an oven and allowed to dry at 50°C for 16 hours and the dry weight was determined using the formula: % Dry matter = (Weight of the dried peels/fiber ÷ wet weight of the peels/fiber)* 100.

The aqueous extract was prepared by grinding approximately 100 grams of both the dried peel and fiber and dissolving them in 850ml of distilled water as described by Ndikau et al,. (7). The beakers containing the dissolved powder were labeled and placed on a shaking water bath and heated at a temperature of 75°C for 10-15 minutes to increase the yield of the water-soluble polyphenols. The extract was allowed to cool before filtration using a vacuum filter to recover particulate-free extract.

Synthesis of silver nanoparticles

Silver nitrate (0.1M) was prepared by dissolving 1.6987grams of silver nitrate in 100ml distilled water (7). The solution was reacted with 10ml of the jackfruit peel and fiber extracts, respectively. The reaction mixtures were stirred using magnetic stirrers for a minimum of 4 hours until the color of the solution changed from yellow to brown (7). To confirm the reduction of silver to silver, silver nanoparticles were prepared by reacting the 0.001M silver nitrate solution with 1% trisodium citrate.

Characterization of silver nanoparticles

UV-VIS spectral analysis

Approximately 1ml of the jackfruit peel extract-silver nanoparticle sample was diluted with distilled water and then placed in a quartz cuvette with a 1cm path length and inserted in a UV-Vis spectrophotometer in the wavelength of 300-700nm to obtain the spectra of the jack fruit peel extract (7).

Voltammetry analysis

A blank comprising of solution of phosphate buffer and distilled water was run in the system. This was followed by running the analyte whereby phosphate buffer mixed with a few drops of jackfruit peel-$AgNO_3$ was added into the electrochemical cell and placed the electrodes in the solution. The parameters were entered into the system the initial potential and final potential were -600mV but the switching potential was +1300mV. It had 3 complete cycles/segments and varied scan rates from 40 to 200mV/s.

FTIR spectra analysis

When working with a liquid sample, the solid sample accessory of the FTIR machine was removed and replaced with a liquid sample accessory. A pipette was used to draw the synthesized silver nanoparticles solution of the peel and fiber and placed it on the crystal portion of the FTIR. The

liquid sample accessory was pushed downwards to come into contact with the liquid solution and a graph with peaks was generated. This was repeated for the synthesized silver nanoparticles solution of the fiber extract.

Evaluation of antimicrobial activity

The synthesized nanoparticles were tested for antimicrobial activity using the agar well diffusion method according to the CLSI guidelines and the zone of inhibition was measured (8). To evaluate the antimicrobial activity, Mueller-Hinton agar medium was prepared to culture the bacteria. To prepare the Mueller-Hinton agar, 38 grams of the Mueller-Hinton powder were placed into a 1000ml glass bottle and 1000ml of distilled water was added and the glass bottle was closed and placed in an autoclave at 121°C for one hour. After one hour, it was removed and placed in cold water making sure that it does not solidify and then the liquid media is poured onto Petri dishes, on cooling, the Petri dishes were covered and placed in the fridge. Fresh cultures of *S. aureus* and *E. coli* were obtained from KEMRI and stored in the USIU refrigerator. 5ml of normal saline was measured into two test tubes and using a swab, the *S. aureus* and *E. coli* from their respective Petri dishes were swabbed and placed into the 5ml normal saline. Once the solution containing the microorganisms was made, a swab was used to spread the bacteria on the Mueller-Hinton agar using aseptic techniques. This was followed by digging wells on the plates using pipettes. Solutions of silver nanoparticle samples to be tested (100 microliters) were then placed in wells. All the agar plates were incubated for 24 hours at 37 °C and after 24 hours, the antibacterial activity was determined by measuring the zone of inhibition around the wells containing the samples. For each sample, the experiment was performed in triplicates and an average of the zone of inhibition was calculated (9).

Evaluation of synergistic/antagonistic activity with common antimicrobials

The action of biogenically synthesized nanoparticles with better antimicrobial activity in combination with amoxicillin trihydrate and flucloxacillin, which are antibiotics with reported resistance against *S. aureus* and *E. coli*, respectively was evaluated. The antimicrobial activity was tested using the agar well diffusion method according to the CLSI guidelines (8) and the zone of inhibition was measured as described previously. Ten (10) milligrams of each of these antibiotics were dissolved in 1000ml of distilled water and added into the solution of the synthesized silver nanoparticles of the jackfruit peel and fiber separately (9).

Results and Discussion

Preparation of jackfruit extracts

Drying jackfruit was essential because it is has large amouts of sugars that could lead to spoilage upon storage. Therefore oven drying was the fastest way to dry the fruit. The temperatures of the oven were kept to a minimum of 50°C because according to research done previously by Adan *et al.*, they were extracting phytochemicals from jackfruit peel and fiber waste and they used temperature of 105°C (6). This was mainly done to extract a large amount of phytochemicals. In this study, a small amount of polyphenols was required as they are the reducing agents and therefore temperatures of 50°C were suffient to extract a good amount of phytochemicals (polyphenols). The jackfruit extract was prepared as shown in f**igure 1** where it yielded a wet weight of 4173 grams and 1714 grams for the peels and fibers, respectively. The dry weight of the jackfruit peels and fibers was determined to be 56% and 49%, respectively.

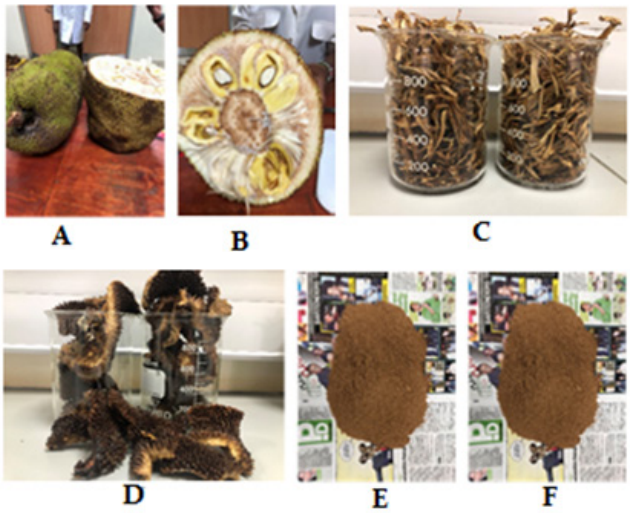

Figure 1. A-Whole fruit, B-Cut open fruit, C-Dried fiber, D-Dried peel, E-Jackfruit peel powder,

F-Jackfruit Fiber powder

Silver nanoparticles synthesis

When the peel extract and the fiber extract were reacted with silver nitrate solution for 4-5 hours, the color changed from yellow to brown as shown in **figure 2**. This indicated a reduction of the silver ions resulting in the formation of silver nanoparticles (7). The reduction was caused by the phytochemicals such as polyphenols, tannins, flavonoids, triterpenoids, and anthocyanins which acted as reducing, stabilizing, and capping agents in the biogenic synthesis of silver nanoparticles (6).

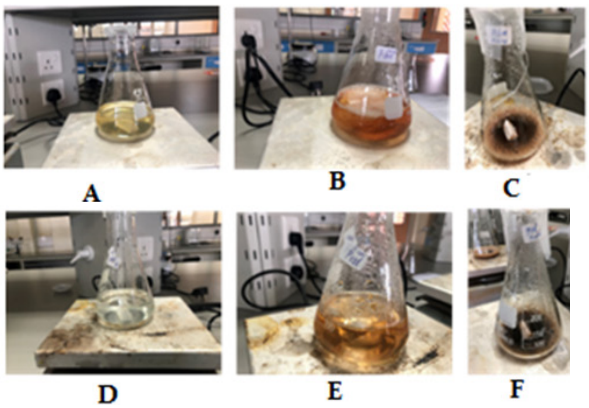

Figure 2. A-Fiber nanoparticles at time t=0, B-Fiber nanoparticles after 2 hours, C-Fiber nanoparticles after 5 hours, D- Peel nanoparticles at the start, E-Peel nanoparticles after 2 hours, F-Peel nanoparticles after 5 hours.

The synthesized silver nanoparticles were further confirmed by UV-VIS spectrophotometer which indicated an optimum peak at 445nm for the AgNPP and 427nm for the AgNPF as shown in **figure 3**. The results were partly similar to results reported by Badi *et al*., where silver nanoparticles were found to appear between 390-440nm (10). This indicates that the phytochemicals in the peel and fiber extract reduced the silver nitrate to form silver nanoparticles. Chemical synthesis gave a peak of 430nm while the peel alone or fiber alone did not show a reduction of silver to silver ions. The high absorbance from the chemical synthesis than that of the peel and fiber nanoparticles indicates a greater concentration of silver nanoparticles. This means that more reduction occurred in the chemical synthesis than in the biogenic synthesis and this is mainly due to a lower amount of reducing and stabilizing agents in the plant material hence reduction is much lower when reacted with silver nitrate solution (11).

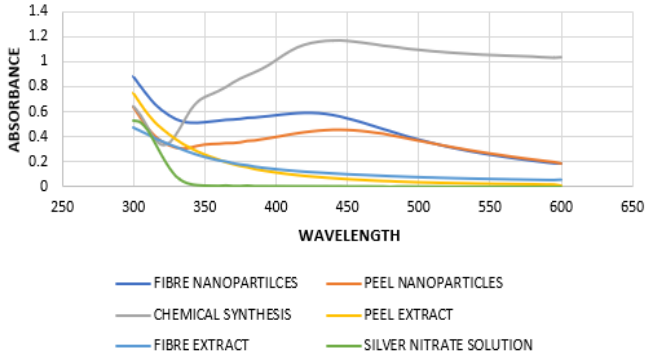

Figure 3. UV-VIS absorbance of peel extract, fiber extract, peel nanoparticles, fiber nanoparticles, and silver nitrate solution.

Characterization of Silver Nanoparticles

FTIR spectra analysis

The jackfruit peel and fiber have several phytochemicals present. The peel mainly consists of flavonoids, triterpenoids, tannins, alkaloids, and saponins whereas the fiber consists of flavonoids, tannins, polyphenolic compounds, and anthocyanins (12). Both the powders and synthesized silver nanoparticles were analyzed through the FTIR at the range of 4000 to 500 cm-1. The powder of the peel and fiber showed several peaks as shown in **figure 4** and **figure 5**. For the peel powder, the peak at 3337 cm^{-1} represents an OH group whereas the peak at 2932 cm^{-1} represents the C-H bond of methylene and methyl group. At 1719 cm^{-1}, it represents the C=O of a carboxylic acid group and an ester group of pectin whereas the peak at 1603 cm-1 represents the C=O stretching bond of carboxylic acid (13). Furthermore, the peak at 1375 cm^{-1} represents the CH3 bond of an ether group. In addition, the peaks at 1022 cm^{-1} and 587 cm^{-1} represent an ether group and it represents the SO3 stretching and C-O stretching respectively (13).

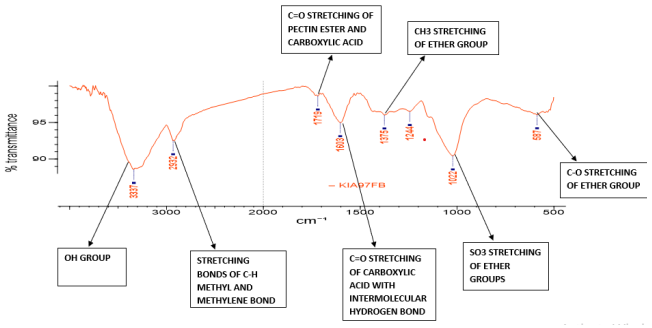

Figure 4. FTIR analysis of powdered peel extract

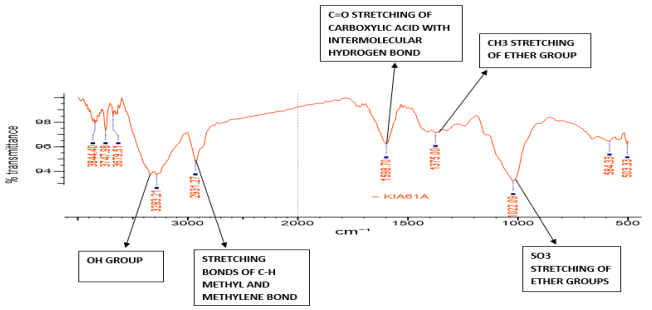

Figure 5. FTIR analysis of powdered fiber extract

For the fiber powder, it showed several peaks, and each represents a specific functional group. For the peak at 3283 cm-1, it represents the OH functional group whereas the peak at 2931 cm^{-1} represents stretching C-H bonds. In addition to that, a peak was visible at 1598 cm^{-1} and it represents the C=O bond of carboxylic acid. The other peaks present represent the ether group and to be specific, the peak at 1375 cm^{-1} represents the CH_3 bond whereas the peak at 1022 represents the SO_3 stretching bond (13).

The nanoparticles formed by the peel and fiber showed similar graphs and peaks indicating that silver nanoparticles were formed mainly through reduction. The reduction in the number of peaks is seen in **figure 6A** for the peel nanoparticles and **figure 6B** for the fiber nanoparticles.

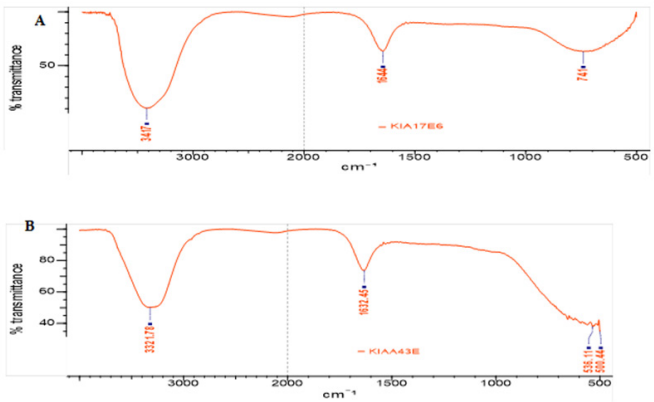

Figure 6. FTIR analysis of synthesized silver nanoparticles from (A) peel extract and (B) fiber extract

For both the peel and fiber, the peaks were formed at similar positions. For the peel, there were three peaks at 3417 cm^{-1}, 1644 cm-1, and 741 cm^{-1} whereas for the fiber, the most prominent peaks are found at 3321 cm^{-1}, 1632 cm^{-1}, 536

cm-1, and 500 cm^{-1}. In comparison with the peaks of the powder, the number of peaks has reduced significantly, and the carbonyl and hydroxy groups have shifted. As a result, we can say that the major components of the extracts, mainly the flavonoids, tannins, and polyphenols were capped onto the surface of silver nanoparticles and showed certain peaks on the FTIR spectrum.

Voltammetry analysis

There were no peaks for the blanks on the voltammogram as the lines remained close to the baseline indicating that none of them had any electrochemical species present. In addition to that, the peel extract and fiber extract were allowed to run alone to see the peaks on the voltammogram and these samples showed no peaks indicating that no electrochemical species were present in the peel and the fiber. The parameters used for the peel and fiber nanoparticles were both different where for the peel, the initial potential, and final potential is -600mV, the switching potential was +500mV, segments were 3 and different scan rates such as 50,100 and 150mV/s were used. On the other hand, for the fiber, the initial potential and final potential are -175mV, the switching potential was +700mV, segments were 3, and different scan rates such as 80,160, 170, 200, and 250mV/s were used as shown in **figure 7A and 7B**. For the peel, the minimum scan rate was 50mV/s whereas the maximum scan rate was 150mV/s and at this maximum scan rate, it showed a distinct cathodic peak at 0.47V and anodic peak at 1.01V. For the fiber, the minimum scan rate was 80mV/s whereas the maximum scan rate was 250mV/s and at this maximum scan rate, it showed a distinct cathodic peak at 0.73V and anodic peak at 1.55V.

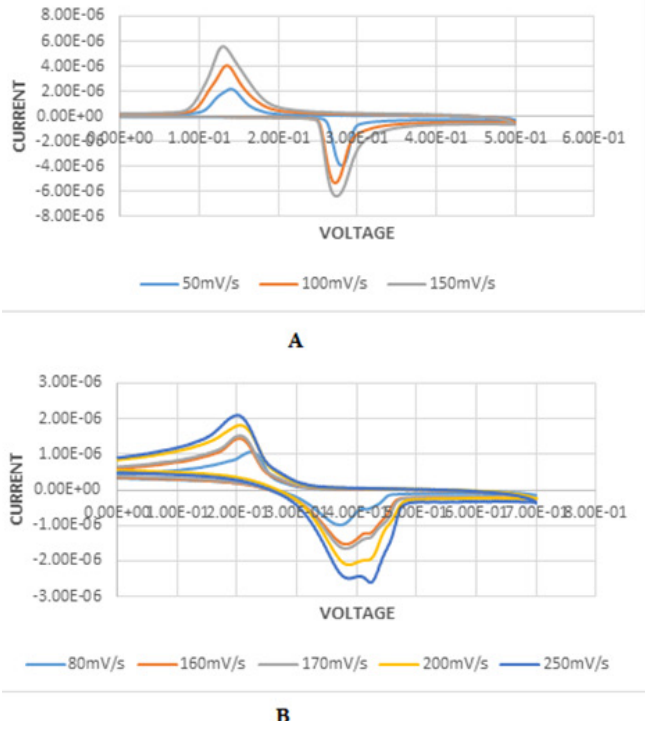

Figure 7. Voltammetry analysis for (A) peel nanoparticles and (B) fiber nanoparticles

Evaluation of Antimicrobial Activity of synthesized silver nanoparticles

The silver nanoparticles synthesized from jackfruit peel and fiber demonstrated antibacterial activity against gram-positive *S. aureus* and gram-negative *E. Coli* as shown by the diameter of the zone of inhibition presented in **Table 1**.

The control used in the experiment was the peel extract, the fiber extract and the silver nitrate solution and we can say that the jackfruit extracts had a greater effect on *Staphylococcus aureus* than *Escherichia coli* bacteria since the average zone of inhibition for both the peel and fiber was larger. On the other hand, for silver nitrate solution, the average effect was much higher against *Escherichia coli* bacteria as compared to *Staphylococcus aureus* bacteria meaning that it was more effective on *Escherichia coli* bacteria. The antimicrobial activity from the peel and the fiber is contributed from the phytochemicals present and the peel mainly contains flavonoids, tannins, saponins, proteins and triterpenoids whereas the fiber contains polyphenols, anthocyanins, and carboxylic acid such as pentanoic acid and hydroxy-propionic acid (14). For both the peel and the fiber, polyphenols form a large proportion of the phytochemicals and the polyphenols have been shown to have antibacterial properties (14). The mechanism is by suppression of virulence factors in the microbes. For example, it can inhibit formation of biofilms or inhibiting adhesion of host ligands or toxins in the bacteria being neutralized. According to research, it has been noted that the phenolic compounds attribute to the configuration of the structure and this in turn results to the antibacterial activities. This is also demionstrated from the findings here attributed to the OH group and the degree of polymerization (6)

Nanoparticles from the peel extract demonstrated better antimicrobial activity towards *S. aureus* than *E. coli* with a zone of inhibition at 16.0 and 11.7 mm, respectively, while the nanoparticles from fiber demonstrated better antimicrobial activity against *E. coli* with an inhibition zone of 21.8 mm. This can be interpreted that either of the plant extracts can be used for the green synthesis of silver nanoparticles and the antibacterial activity of the nanoparticles is not affected by the difference in the bacterial wall though the zone of inhibition is different. Previously, nanoparticles prepared from *Carduus crispus* have been shown to have antibacterial activity against both gram-negative *Escherichia coli* (5.5 ± 0.2 mm to 6.5 ± 0.3 mm) and gram-positive *Micrococcus luteus* (7 ± 0.4 mm to 7.7 ± 0.5 mm) (15). According to previous research, the main difference in activity between gram-negative bacteria and gram-positive bacteria is the structure of the cell wall. Generally, gram-positive bacteria have a thick peptidoglycan layer whereas gram-negative bacteria have a thin peptidoglycan layer. It has an additional layer of lipopolysaccharides and the combination of these can have an impact on how the silver nanoparticles affect it (16). Gram-negative bacteria have a single thin layer of

peptidoglycan and therefore allow easy penetration of the silver ions whereas gram-positive bacteria have a thicker layer of peptidoglycan and this forms a barrier and thus preventing entry of the silver ions. This means that gram-negative bacteria are more prone to damage compared to gram-positive bacteria (17).

Table 1. The zones of inhibition produced by silver nanoparticles prepared from jackfruit extracts and their combination with amoxicillin and flucloxacillin against *S. aureus* and *E. coli*

Sample	Zone of Inhibition (mm)	
	S. aureus	*E. coli*
Peel Solution	21.67±2.1	18.33±1.2
Fiber Solution	22.67±3.1	18.67±1.5
Silver Nitrate Solution	18.67±3.2	19.33±3.1
Peel Nanoparticles	16.0±4.6	11.7±2.1
Fibre Nanoparticles	13.7±2.3	21.8±5.1
Flucloxacillin	17.3±0.6	19.3±2.1
Amoxicillin	25.7±0.6	21.0±0.0
Amoxicillin + Peel Nanoparticles	30.0±1.0	18.0±3.6
Amoxicillin + Fiber Nanoparticles	30.3±0.6	14.7±0.6
Flucloxacillin + Peel Nanoparticles	11.8±0.6	12.7±0.6
Flucloxacillin + Fiber Nanoparticles	19.7±0.6	16.0±1.01

Various mechanisms that contribute to the antimicrobial effect of silver nanoparticles have been proposed including cytoplasmic membrane disorganization and the consequent leakage of various biomolecules such as amino acids, protein, and carbohydrates, inhibition of various essential enzymes, and formation of pores on a membrane that result in diffusion of nanoparticles into the cell where it binds with sulfur and phosphorous proteins, leading to inactivation of DNA and proteins (18). One of the main advantages of using silver nanoparticles over conventional chemical antibiotics is mainly because conventional chemical antibiotics cause multidrug resistance (16). The antibiotics mainly show effectiveness if the surface of the drug or its metabolites binds to the microbes and over time, the microbes develop resistance due to this impact. Silver nanoparticles are much more effective as the microorganisms are unlikely to develop resistance to the synthesized nanoparticles (16).

Evaluation of synergistic/antagonistic activity with common antimicrobials

The controls used in the study were amoxicillin and flucloxacillin antibiotics and results indicated that the average zone of inhibition was much larger for amoxicillin on *Staphylococcus aureus* than on *Escherichia coli* whereas the average zone of inhibition of flucloxacillin is much larger in *Escherichia coli* than in *Staphylococcus aureus*.

For *Staphylococcus aureus* bacteria, it was observed that, the combination of amoxicillin and the synthesized nanoparticles of the peel and fiber are more effective than the combination of flucloxacillin with the synthesized nanoparticles of the peel and fiber. For *Escherichia coli* bacteria, we can say that the combination of amoxicillin and the synthesized nanoparticle of the peel is more effective than the combination of flucloxacillin and the synthesized nanoparticles of the peel. On the other hand, the combination of flucloxacillin with the synthesized nanoparticles of the fiber is more effective than the combination of amoxicillin and the synthesized nanoparticles of the fiber. Therefore, the results indicated that the combination of amoxicillin with peel nanoparticles and combination of flucloxacillin with fiber nanoparticles is more effective against *Escherichia coli* bacteria.

The combination of amoxicillin with the peel and fiber nanoparticles showed a synergistic effect since the average zone of inhibition was higher than when antibiotics were used alone as shown in **Table 1.** On the other hand, the combination of flucloxacillin with the peel and fiber nanoparticles had an antagonistic effect since the average zone of inhibition was lower than that of the antibiotics alone.

A study by Lee and coworkers indicated that silver or gold nanoparticles, when used in combination with antibiotics, show synergistic effects and they have been useful against *S. aureus, E. coli, A. baumannii, E. faecium*, and *Pseudomonas. aeruginosa*. They both work together by penetrating the cell membrane of the bacteria and inside the cell, they interfere with important molecular pathways (19). The combination of nanoparticles and antibiotics has been shown to be effective against antimicrobial resistance and in addition to that, it increases the antimicrobial effects. The common nanoparticles like silver, gold, or zinc oxide can be combined with antibiotics like clindamycin, vancomycin, ampicillin, or erythromycin and the synergistic effects can be utilized against *P. aeruginosa, E. faecium*, and methicillin-resistant *S. aureus* (MRSA) (19). A study by Usama and coworkers indicated that the combination of silver nanoparticles with certain antibiotics such as oxacillin, or neomycin resulted in an antagonistic action as the zone of inhibition decreased in comparison to when antibiotics were used alone on *E. coli, S. aureus*, and *Salmonella spp* (20).

Conclusion

Silver nanoparticles were synthesized from the peel and fiber of jackfruit and characterized, and UV-VIS spectroscopy that confirmed the results with absorbance peaks observed between 390-440nm. Furthermore, FTIR confirmed the presence of silver nanoparticles since two major peaks were formed at 3300cm^{-1} and 1600cm^{-1} for both extracts. In addition, voltammetry did show a redox reaction taking place indicating that silver nitrate was reduced, and silver nanoparticles were being formed. The peel and fiber nanoparticles confirmed an antibacterial effect and when combined with antibiotics, it confirmed synergism on the *Staphylococcus aureus* bacteria and antagonism on *Escherichia coli*.

The main limitations for this study were the cost since some of the reagents like silver nitrate and bacterial cultures are expensive. Furthermore, the study proved to be time consuming since the fruit itself is not readily available and also the cutting, to extraction as well as characterization took much more time than anticipated.

Recommendations

There are few notable recommendations for future research: for example, instead of using an oven to dry, air drying could be an alternative method to see whether there is a difference between the two methods of drying. Also, a suggestion of using raw jackfruit peel and fiber instead of drying the fruit to see whether drying has an impact. Another recommendation is to evaluate synergistic/antagonistic activity of the synthesized silver nanoparticles with common antibiotics with reported resistance on *Staphylococcus aureus* and *Escherichia coli*.

Conclict of Interests

Authors declare no conflict of interest.

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A purview of herbal therapies deployed in the traditional management of sickle cell disease in Kwara – Nigeria

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Abstract

Background: Over 150,000 children are born with Sickle cell disease (SCD) annually in Nigeria, with complications ranging from severe pains to death. Plants materials have been used for medicinal and nutritional purposes over the years. Many natural medicines though used in various Nigerian communities are yet to be sufficiently identified and studied for potential standardisation and development as sources of new drugs which will be of immense benefit to humanity. This study is geared towards contributing to the existing body of knowledge on natural medicines used to manage SCD in Nigeria, towards standardising and further developing them for potential clinical use.

Aim: The study aims to survey four local government areas of Kwara State, Nigeria, for the medicinal plants used in managing sickle cell disease by traditional medical practitioners.

Method: Data relating to the identities of the natural medicines, the nature of ailments, and the demographic information of participating traditional medical practitioners were collected using interviewer-administered, semi-structured questionnaires.

Findings: Thirty-seven respondents were interviewed (62% male and 38% female). Majority of the respondents were fairly educated (mostly primary and secondary school graduates) with an average of 15 years of experience as traditional healers. Over twenty-five plants with twenty-five genera and twenty families were mentioned as being used in managing sickle cell disease. The practitioners do not usually determine treatment duration. Treatment was mostly monitored by patients' verbal responses and physical examinations. The traditional medical practitioners identified several herbs used in sickle cell disease in Kwara State, Nigeria.

Conclusion: From the responses obtained, the need for improvement in therapy standardisation and drug handling of medicinal plants is dire. Many plants used in treating SCD have been revealed in this study. Therefore, it is highly recommended that scientific validation of the activities of the herbs should be done as a follow-up.

Key Words: *Traditional, Practitioners, Herbs, Medicines, Sickle Cell.*

Introduction

Sickle cell disease (SCD) is an inherited genetic haematological disorder characterised by mutated haemoglobin. It affects millions of people across the globe, with a particularly high birth prevalence in sub-Saharan Africa. It is a common genetic disease and of utmost public health importance in sub-Saharan Africa (1). The sickle haemoglobin (HbS) is an abnormal variant of the adult haemoglobin caused by a mutation. In patients with SCD, there is a reduction in the oxygen-carrying ability of the red blood cells, potentially leading to episodes of microvascular occlusion, ischemia, and severe pains(2). The consequences can often be life-threatening. The global spread of SCD has been connected to the natural protection against malaria in heterozygous individuals for the mutation (3). This selective advantage over the years has resulted in the distribution of HbS mutations with some focus around the tropics. Unfortunately, many of these countries in the tropics are not sufficiently equipped with the necessary resources to provide the array of complicated care needed by SCD patients; prognosis, therefore, has remained typically poor (4).

Nigeria, the most populous African nation in the world, has the highest prevalence of SCD in sub-Saharan Africa (5). Although there has been some significant scientific progress in understanding the disease recently, the prevalence of sickle cell trait still ranges between 10 and 45% in various parts of sub-Saharan Africa (6). SCD affects about 2 to 3% of the Nigerian population (5). Data from a large retrospective study by Nwogoh and co-workers (7) in Benin City, Nigeria, revealed an SCD prevalence of 2.39% and a carrier rate of about 23%. With Africa and Asia generally considered the birthplace of sickle cell mutation, the need for concerted efforts to reduce this burden is dire. African research communities must take charge and explore more therapeutic alternatives.

Bone Pain Crisis (BPC) is a consistent presentation of SCD.(8) Excruciating pains result from the activation of nociceptive

afferent nerve endings in the ischemic bones (9). In its early stage, SCD can also induce dactylitis in the small bones of the hand or foot, marked by diffuse swelling over the involved area (10). This may subside spontaneously within 10-14 days and is rare after 2-3 years of life.

Traditional medical practitioners in Nigeria and broadly, West Africa, have long operated with sophistication. Ameh et al. reported that various communities within the country had already associated certain infections and conditions like high fever and dysentery with their parasites or vectors (11,12). Numerous plants have been used to treat sickle cell anaemia in Nigerians as reported in literature (13,14). Among those that have been used are clove (*Eugenia caryophyllata*), grains of paradise known among locals as alligator pepper (*Aframomum melegueta), Sorghum bicolor,* and *Pterocarpus sp*.(12) Additionally, earlier research efforts have identified phytochemicals obtained from *Carica papaya*,(15,16) Garlic,(17) *Cajanus cajan*,(18) and *Hymenocardia acidai*,(19) among other plants, all of which have been used traditionally to treat sickle cell anaemia in Nigeria (12). Based on these findings, it is clear that the potentials of natural products are limitless, justifying the need to explore them more extensively as sources of drugs in the management of sickle cell disease.

During the 1970s, studies at Ibadan and Ife described the first series of herbal remedies for SCA(20). More recently, in 2001, before the franchise to produce NIPRISAN® was licensed to a US drug firm, the National Institute for Pharmaceutical Research and Development (NIPRD), had three other promising formulations (20). According to the World Health Organization (WHO), 80% of the emerging world's population depends on natural therapies (21, 22). Herbal medicines include herbs, herbal materials, preparations, and finished herbal products containing parts of plants or other plant materials as active ingredients and have been used for medicinal and nutritional purposes over the years (23, 24). Natural medicines used among various Nigerian communities must be identified and studied for potential standardisation and development as sources of new drugs of immense benefit to humanity.

Aim/Objective

This survey formed a basal template for exploring readily available herbal sources of drugs for sickle cell disease. It aimed to survey four local government areas of Kwara State, Nigeria, for the medicinal plants used in the management of sickle cell disease by traditional medical practitioners. It also looked at providing insights on the components of traditional medical practice -including documentation, referral, follow-up, and therapy monitoring, within the sampled local government areas and their determinants.

Methodology

Study Area

This survey was conducted in four local government areas of Kwara State - Nigeria: Ifelodun, Asa, Ilorin-East, and Ilorin-West. Kwara state is situated in the North-Central geopolitical zone of Nigeria, with a population of 777,667 as at the last census in 2006. The state has a rich and diverse cultural representation, as seen in the geographical representation of the study area (**Figure 1**).

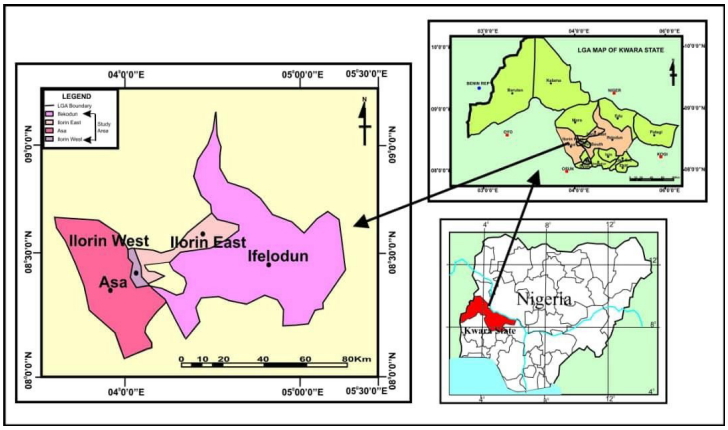

Figure 1. A geographical representation of the study area

Study design

The study was a cross-sectional descriptive design. Traditional medical practitioners (TMPs) residents in specific areas of Kwara State were interviewed for information on the traditional management of sickle cells.

Inclusion criteria

Only traditional medical practitioners who resided in Asa, Ifelodun, Ilorin East, and Ilorin West local Government areas were voluntarily enrolled.

Exclusion criteria

i. Individuals who were not traditional medical practitioners in the focus local government areas

ii. Individuals amongst the traditional medical practitioners who refused to give consent as volunteers for the study.

iii. Individuals who were deaf and could not communicate verbally.

Sampling technique

The sampling method used was purposive. Traditional medical practitioners were deliberately targeted as subjects of the research for the wealth of information and experience they possess on the intended study subject of traditional remedies used in their communities to manage sickle cells and related ailments. All identified and willing respondents who gave informed consent were recruited for the study.

Sample size determination

A total of thirty-seven traditional medical practitioners were enrolled for this study across the four local government areas. This number represented the total number of practitioners in these areas that consented and were available to participate in the study. As such, the number 37 is a representation of all the practitioners identified in the study zones, making partial sampling unnecessary.

Preparation of questionnaires

The used questionnaires were self-designed by the

researchers based on the range of information required for the study and the peculiarities of the focus population. Prepared questionnaires were semi-structured and divided into two sections: Part A collecting demographic information, and Part B on responses relating to traditional medicines and approaches in sickle cell management. Questionnaires were tested for appropriateness and suitability using four subjects: one from each local government area.

Data collection

Data relating to the identities of the natural medicines, nature of ailments, demographic information of participating traditional medical practitioners and users was collected using interviewer-administered, semi-structured questionnaires. Questionnaires were administered by the researchers with assistance from two research assistants who had been trained for two weeks by the researcher on methods of approach, consent seeking, and techniques of data collection and documentation, before being deployed to the field. Data collection was done for four weeks across the four local government areas of Kwara State (Ilorin west, Ilorin east, Ifelodun and Asa), Nigeria. The questionnaires were administered at the practice site of the traditional medical practitioners in these focus areas.

Taxonomic identification

Authentication of plants identified from the study was done at the herbarium of the Department of Plant Biology, University of Ilorin.

Data Analysis and Statistics

The questionnaire responses were coded and then entered as data into relevant computation software. Data was organised and visualised using the Microsoft Excel software. Descriptive statistics were presented as frequency, percentages, mean and standard deviations. Statistical comparison of some of the demographic responses provided for associations with responses on sickle cell management by traditional medical practitioners was done using the Chi-Square statistical tool on the IBM SPSS (V23) software package.

Ethical Issues

Ethical approval for this survey was obtained from the Nigerian National Code for Health Research Ethics (under the Collaborative Training Initiative Program of the West African Bioethics Training Program) with reference numbers of the two researchers being as follows: CITI NHREC Ethics Record ID: Adeola Kola-Mustapha 25568449 and Yusuf Ghazali 24781406. The research, which was a questionnaire-based survey, did not involve utilising human or animal samples at any point. The data collected were further analysed anonymously. Informed verbal consent was given by all respondents and was witnessed by the Head of the TMPs in the study area.

Results

The study involved thirty-seven (37) Traditional Medicinal Practitioners (TMPs). The TMPs comprised 62% males and 38% females, as presented in **Figure 2**.

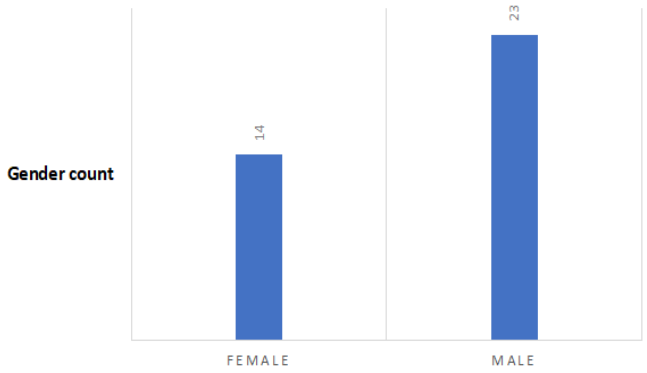

Figure 2. Gender distribution of respondents

The educational status of the TMPs ranged from none to tertiary education, as presented in **Figure 3**. Majority of the respondents had received no formal education, while the rest got through primary education, with a few obtaining a secondary education as their highest formal education.

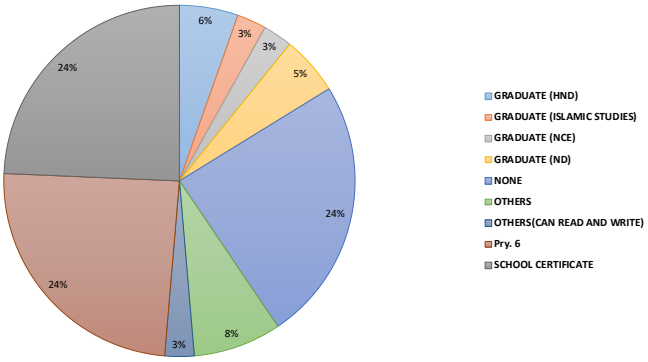

Figure 3. Educational Status of Traditional Medical Practitioners (TMPs).

Most of the respondents (89.2%) got their herbal training from their families, while others paid for it under the apprenticeship scheme, as presented in **Table 1**. A majority of the respondents (62%) had been practicing for between 11 and 20 years. The years of experience as TMPs of the respondents are presented in **Table 2**.

Table 1. Training of Practitioners

Training	Distribution	Percentage (%)
Apprenticeship (10 Years)	1	2.70
Apprenticeship (5 Years)	1	2.70
Apprenticeship (5 Years)	1	2.70
Family	33	89.20
Family and Apprenticeship (6 Years)	1	2.70

Table 2. Cumulative Years of Practice of Practitioners.

Years as TMP	Distribution	Percentage (%)
0 – 10 years	6	16.22
11 - 20 years	23	62.16
21 – 30 years	8	21.62

The data obtained shows that most TMPs in the sample population were younger than 20 years of age while a small minority (3%) were older than 60 years. The age distribution of the respondents is presented in **Figure 4**.

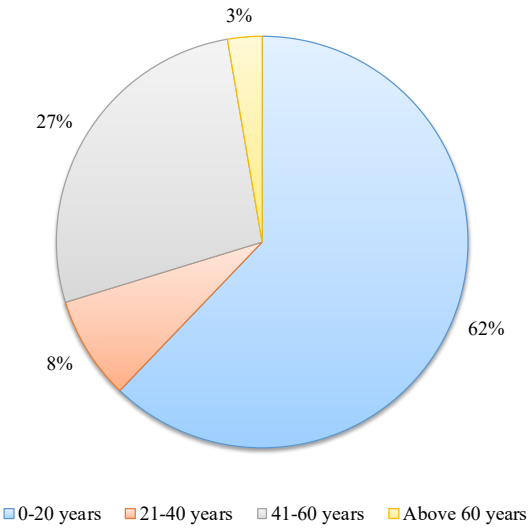

Figure 4. Age Distribution of Respondents

As presented in **Figure 5**, most of the respondents (76%) believed that SCD could be treated between 0-3 weeks

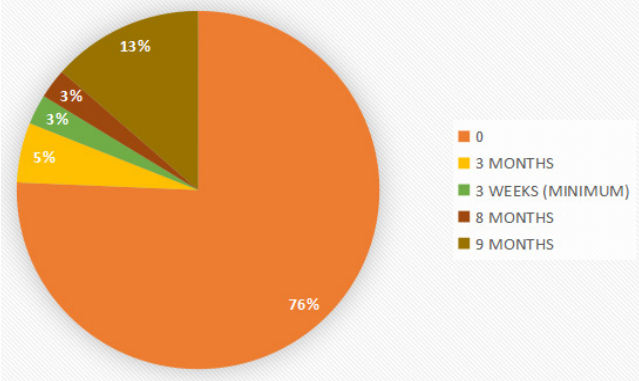

Figure 5. Typical Duration of Herbal Therapy

The types of records maintained by the TMPs are presented in **figure 6**. About 86% of the respondents reported that they do not routinely keep book records of their patients and their clinical data.

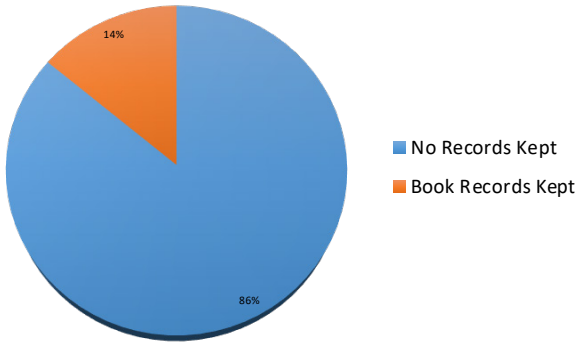

Figure 6. Types of Record Kept by Practitioners

The plants identified by the respondents as useful in managing SCDs are presented alphabetically (botanical names) alongside the voucher numbers (authentication) in **Table 3**.

Table 3. Plants Identified as Used in Sickle Cell Therapies

Local name	Botanical name	Common names	Family name	Voucher Numbers from Authentication
Mafowokanomomi	*Alstonia boonei*	Starbur, Bristly starbur	Asteraceae	UILH/005/1153/2022
Emi isu	*Bryophyllum prinatum*	Goat weed	Asteraceae	UILH/021/140/2022
Alubosa elewe	*Allium ascalonicum Linn.*	Wild onion	Amaryllidaceae	UILH/011/1335/2022
Ahun	*Alstonia boonei*	Stoolwood, Pattern wood	Apocynaceae	UILH/006/1035/2022
Abamoda	*Bryophyllum prinatum*	Air plant, Life plant	Crassulaceae	UILH/019/909/2022
Pawpaw	*Carica papaya*	Pawpaw	Caricaceae	UILH/001/945/2022
Ewe rere abo	*Cassia occidentalis*	Coffee senna, Antbush	Fabaceae	UILH/015/885/2022
Ogede odo	*Crinum jargos*	Crinum Lily	Amaryllidaceae	UILH/003/1022/2022
Egun eja (plant)	*Diospyros monbuttensis*	Yoruba ebony	Ebenaceae	UILH/009/013/886/2022
Abiwere	*Eragrostis tremula*	Japanese lovegrass	Poaceae	UILH/027/104/2022
Igi obo	*Erythrophleum guineense*	Ordeal tree, Sasswood tree	Fabaceae	
Oro adete	*Euphorbia poissoni Pax.*	Spurge	Euphorbiaceae	
Ipin	*Ficus exasperate*	Sandpaper tree, White fig tree	Moraceae	UILH/017/1296/2022
Owu akese	*Gossypium barbadense*	West Indian Cotton	Malvaceae	UILH/012/977/2022
Lapalapa	*Jatropha curcas Linn.*	Physic nut	Euphorbiaceae	UILH/020/978/2022
Odundun	*Kalanchoe crenata*	Never die	Crassulaceae	
Yewuru	*Luffa cylindrical*	Sponge gourd, Dishwash gourd	Cucurbitaceae	UILH/013/523/2022
Ege	*Manihot esculenta*	Manoc, Bitter cassava, Tapioca	Euphorbiaceae	UILH/018/1094/2022
Ogede	*Musa paradisiaca*	Banana	Musaceae	UILH/002/138/2022
Egbesi	*Nauclea Africana / Nauclea latifolia*	Nauclea African peach	Rubiaceae	UILH/016/1412/2022
Efinrin	*Ocimum basilicum*	Scent leaf, Balsam basil	Lamiaceae	UILH/004/1354/2022
Idi ipete	*Securidaca longepedunculata*	Violet tree	Polygalacea	UILH/008/192/2022
Tomati	*Solanum lycopersicum*	Love apple, Tomato	Solanaceae	UILH/010/1350/2022
Ewuro	*Vernonia amygdalina*	Bitter leaf	Asteraceae	UILH/007/1023/2022
Eru alamo	*Xylopia aethiopica*	African pepper, Guinea pepper	Annonaceae	UILH/014/1089/2022

The methods by which the sampled TMPs assess patients' response to the SCD therapy are elucidated in **Table 4**. Over 80% of the respondents reported monitoring patients through verbal responses and physical examinations done within 10 days of commencing therapy.

Table 4. Methods of Assessing Response to Therapy

Monitoring	Distribution	Percentage %
Patient's Verbal Response	3	8.11
Patient's Verbal Response, Physical Examination (Within 10 Days)	31	83.78
Patient's Verbal Response, Physical Examination (Beyond 10 Days)	2	5.41
Physical Examination, Medical Check Up	1	2.70
Total	**37**	**100**

The sampled TMPs were asked structured questions regarding their general approach to SCD herbal therapy, and their responses are presented in **Table 5**.

Table 5. Structured Responses to Questions on Traditional Sickle cell Management

Questions	Responses	
	YES (%)	NO (%)
Do you know most of your patients?	5	95
Do you keep records of your clients?	14	86
Do you belong to an Association/Union of Traditional Medical practitioners?	100	0
Do you treat sickle cells or related ailments?	100	0
Do you consider yourself a specialist in sickle cell management?	100	0
Do you have conservation methods for the plants you use?	100	0
Are these medicinal plants used as food?	100	0
Are the medicinal plants used going into extinction?	100	0
Are you aware that some orthodox medicines used in sickle cells are obtained from plants?	100	0
Do you think there is room for improvement in your practice?	100	0
Do you make referrals to the hospital?	43	57

A summary of the inferential statistical analysis conducted to establish correlational relationships between the demographic data obtained from the respondents and various components of their practice is presented in **Table 6**.

Statistical Analysis

Table 6. Testing Relationships between Categorical Variables Using Chi-Square Statistics.

Demographics Compared	Category 1	Category 2	Category 3	Category 4	P values	Remarks
Gender	Duration of Treatment	Referrals to Hospital	Keeping Records	Type of Training	<0.05	Significant (relationship exists)
Level of Education		Referrals to Hospital			>0.05	No significant relationship exists
Level of Education			Keeping Records		<0.05	Significant (relationship exists)
Age				Type of Training	>0.05	No Significant relationship exists
Age		Referrals to Hospital			<0.05	Significant (relationship exists)
Age				Keeping Records	<0.05	Significant (relationship exists)
Type of Training		Referrals to Hospital			<0.05	Significant (relationship exists)
Type of Training			Keeping Records		<0.05	Significant (relationship exists)
Years of Experience as a Practitioner					>0.05	No Significant relationship exists
Years of Experience as a Practitioner	Duration of Treatment	Referrals to Hospital			<0.05	Significant (relationship exists)
Years of Experience as a Practitioner			Keeping Records		<0.05	Significant (relationship exists)

Discusion

There has been a gradual decline in the knowledge and practice of traditional medicine arising from a lack of proper documentation, unwillingness to pass down knowledge, and wide acceptance of orthodox medicine (25). It was observed that a good proportion of the participating practitioners offered treatments without monetary rewards from their patients. This fact suggests that some of these practitioners are in the business out of genuine compassion for their communities and not for commercial gains.

Interests in herbal medicines continue to grow globally; most people are choosing to be treated with natural therapies (26,27). The prospects of availability, affordability, reduced side effects, and resistance have recently favoured the use of herbal medicine in Africa (28,29). Nigerians are actively tending towards herbs and natural medicines in sickle cell management; this can be seen as an indication to intensify efforts and create policies that will coordinate and sanitise the procurement and administration of these natural medicines to minimise the incidences of long-term organ damage and ensure safety in therapy (30,31). Herbal medicines are made up of phytochemicals that can work synergistically or antagonistically (32,33). Based on this, the common belief that all-natural medicines are safe to use is not completely accurate. This phenomenon underscores the significance of investigations like this, where herbal therapies are identified for pharmacological assessment and the establishment of adequate dosage presentations.

A total of twenty-five plant materials belonging to twenty-five genera and eighteen families were mentioned and identified as being used to manage sickle cell disease. The most frequently occurring families are Euphorbiaceae, Asteraceae with two species each, Fabaceae, and Crassulaceae with two species each. This correlated with the work of Panzu (34) and Amujoyegbe (35), who reported Fabaceae and Euphorbiaceae as the most occurring families of plants with anti-sickling properties in Kikwit City of DR Congo and South Western Nigeria, respectively. Twelve of the plants had previously been reported to possess anti-sickling properties by Amujoyegbe (35).

Male to female respondents were 62% to 38% (**Figure. 2**). Gbadamosi (36) also reported that more men than women were involved in traditional medicine. The cause of the difference in this ratio is yet to be established. The majority of the respondents, 76%, had either formal or informal education and could read and write, while 24% had no form of education (**Figure. 3**). Their level of education elucidates the ability of the TMPs to identify and recognise disease symptoms and recommend the appropriate plant to be used.

Knowledge of traditional medicine was mostly observed to be sacred and passed down from generation to generation. 89.2% of the respondent reported that their practices were based on family knowledge, 8.1% underwent a form of apprenticeship that lasted between 5-10 years, while 2.7% had training in addition to family knowledge (**Table 1**). Their knowledge of management of SCD was also assessed from their years of practice, with the least being five years. A significant number of the respondents (62.16%) had between 11-20 years of experience in managing SCD and other ailments (**Table 2**). Only 3% of the respondents were above 60 years, and 97% were below 60 years, with the most reported age being below 20 (**Figure 4**). These observations are indications that younger people are beginning to take up traditional medicine, which implies a means to preserve the knowledge of herbs and herbal therapy.

A close assessment of the duration of therapy, was mostly undetermined as there was no means of follow-up, record keeping, and methods of checking the response to therapy as presented in (**Table 4, Figures 5, and 6**) respectively, show obvious deficiencies. The methods described are mostly ineffective, especially considering the pathophysiology of cancers and sickle cells in the human body. For instance, it is not ideal to measure sickle cell growth or regression without objective data on the status of the affected cells and body tissue. Therefore, a temporary, latent state in treatment may be seen as a completion of therapy by these practitioners in Kwara, Nigeria. This observed pattern is similar to what was observed by Bahal in 2017; in his research on the 'prevalence, patterns, and perceived value of complementary and alternative medicine among cancer patients' in Trinidad and Tobago (37).

The inferential statistical analysis conducted indicated that some demographic factors affect variables in the practice of herbal therapy of SCD among the study respondents. Gender was found to exhibit a correlational relationship with the rate of referrals to hospitals, record keeping, and the type of training received. Level of formal education

obtained as well as age of the TMP, type of training obtained, and years of experience were found to correlate with record keeping practices, as well as impact referrals to hospitals. While these do not necessarily provide adequate basis for conclusions to be drawn, it certainly provides important direction for future research to further explore the determinants of traditional medical practice, especially as it concerns the management of SCD.

Limitations

The study presents with limitations that especially impact its generalisability and necessitates the contextualisation of its findings. Firstly, due to traditional medical practices being tightly linked to cultural norms across various ethnic groups and civilisations, the patterns of herbal medicine use in managing SCD uncovered can hardly be extrapolated to other regions of the country and to TMPs practices nationwide. Consequently, this makes it difficult to confidently propose initiatives to improve the TMP practice on a nationwide scale, without reproducing the study in other geopolitical regions populated by people with distinct cultures from the sample population in this study. Additionally, the methods utilized in this survey limited the data obtained to TMPs and did not account for herbal medicine use by individuals recommended by herbal product vendors, and other sources that did not fall within the scope of this study.

Conclusion

There is a need to sensitise TMPs on effective clinical approaches to assessing disease progression, and record maintenance. It is also pertinent to preserve and document plants with anti- sickling properties as they can serve as useful leads in managing sickle cell disease, which is of immense public health importance in Africa.

Recommendations

From the findings of the study, it is crucial that Traditional Medical Practitioners be retrained and re-educated through their representative associations on more appropriate means of disease monitoring and assessment to bring them to speed with methods of conventional management. The possibility of collaborations with well-established hospitals and laboratories can also be considered. Further research can be conducted to build on the finding of this study to further characterize traditional medical practice, especially concerning the management of Sickle Cell Disease.

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Notes

i. The respondents disclosed the source of procuring the herbs and natural products as bushes and open herbal markets within the state.

ii. p is set at 0.05 for the measure of significance

iii. Null Hypothesis: there is no statistically significant relationship between compared variables.

iv. Alternate Hypothesis: statistically significant relationships exist between compared variables.

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Antidepressant activity of Methanolic Extracts of *Areca catechu Nuts* in Mice

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Abstract

Background: Depressive disorder is one of the common psychiatric disorders which affects more than 20% of the world population. Although there are several antidepressants available today, the present therapeutic arsenal is frequently insufficient, with disappointing results in around one-third of all people treated. Newer and more potent antidepressants derived from conventionally used medicinal plants with known psychotherapeutic potential and examined in a range of animal models are thus required.

Objective: To determine the anti-depressive potential of methanolic extract of *Areca catechu* nut using mice as the experimental model.

Methodology: *Areca catechu* nuts were purchased from parklands market, Nairobi, Kenya. Methanolic extract was prepared by soaking powdered nuts in methanol for 72 hours. The extract was concentrated through rotary evaporation and used for phytochemical and acute toxicological studies. Forced swimming test (FST) and tail suspension test (TST) were used to investigate presence of anti-depressant properties. Over a period of 10 days, mice were administered with methanolic extracts at concentrations of 50mg/kg, 100mg/kg and 200mg/kg, per day and their swim time or activity time monitored over six minutes. Negative and positive control groups were also included. The negative control group received 1ml/kg of normal saline and the positive control group received 10mg/kg of fluoxetine.

Results: Phytochemical screening of the methanol extract confirmed presence of alkaloids, tannins, and phenols. The lethal dose (LD_{50}) of the extract was determined to be 1265 mg/Kg in mice. The TST showed a dose-dependent effect where anti-depressive activity was more pronounced in higher doses compared to lower doses. At a concentration of 50, 100, and 200 mg/Kg, the average mobility time was 25.9±2.0, 57.0±9.1 and 100.0±21.0 seconds, respectively. Fluoxetine (positive control) had the best activity with an average mobility time of 102.9±4.8 seconds. The FST showed the best anti-depressive activity at a concentration of 200 mg/Kg with an average swim time of 100.6±25.5 seconds, while the positive control had an average swim time of 96.3±24.4 seconds.

Conclusions: The *Areca catechu* nut extracts demonstrated antidepressant activity in FST and TST in mice. The antidepressant activity was dose dependent with best activity being observed at a dose of 200mg/Kg/day of the doses studied. Further study is needed to characterize the mechanism of action and elucidate the active ingredient responsible for the antidepressant-like activity.

Keywords: *Areca catechu, methanolic extract, mice, tail suspension test, forced swimming.*

Introduction

Depression, also known as major depressive disorder (MDD), is a mood disorder that results in a lingering sense of melancholy and loss of interest. Other symptoms of MDD include sleep disturbances, such as insomnia or hypersomnia, lack of appetite which could result in weight loss or increased cravings hence weight gain, anxiety, feeling worthless, trouble thinking, suicidal thoughts, and unexplained physical problems [1]. During the Covid-19 pandemic, depression and anxiety were major diseases suffered by all age groups and sexes. Over 300 million people worldwide suffer from depression, which the WHO has identified as a leading cause of disability [2]. Of more concern is that the likelihood of suicide is up to 30 times higher in teenagers with major depressive disorder. In Kenya, depression is a common cause of morbidity amongst university students with a reported general occurrence of moderate depressive symptoms at 35.7% and severe depression at 5.6% [3]. In terms of gender, women were more likely than men to experience depression ranging from mild, moderate, or severe symptoms [4].

The pathophysiology of depression is not well known. However, various studies suggest that depression is because of low levels of serotonin (5-HT) as well as the involvement of fluctuating levels of other neurotransmitters such as norepinephrine (NE), brain-derived neurotrophic factor (BDNF), glutamate and dopamine (DA) [5]. A number of medications are used to treat depression. The success rate for each medication used to treat this disease is roughly 60%. Additionally, many therapies take weeks of treatment before improvements in symptoms and signs can be seen, and antidepressants have a variety of side effects. For instance, the first line of treatment for depression is selective serotonin reuptake inhibitors (SSRI's) such as fluoxetine. Fluoxetine is one of the main anti-depressants prescribed

but poses a variety of side effects like nausea, diarrhea, anorexia, insomnia, somnolence, anxiety, and asthenia [6]. As a result, the high incidence of depression and the fact that a reasonable part of people do not adequately benefit from antidepressants that are currently on the market indicate the need for new therapeutics to treat depression. Consequently, medicinal plant therapies may be effective options in the management of depression with several species demonstrating antidepressant properties in humans through clinical trials [7]. For instance, S-Adenosyl methionine, *Hypericum perforatum L*, omega-3-fatty acids have shown good anti-depressive activity [8].

According to Boucher & Mannan [9], at least 10% of people worldwide chew areca catechu nuts as a recreational drug, making it the fourth most popular addictive narcotic. In South Asia, the Pacific islands, and East Africa, the nut is widely distributed [10]. The main phytochemical present in the areca catechu nut are alkaloids with arecoline and arecaidine being present in high concentrations. According to Liu et al. [11], arecoline stimulates the central nervous system particularly the muscarinic receptors which promotes excitability and improves learning and memory. Arecaidine is a potent competitive gamma-aminobutyric acid (GABA) receptor inhibitor. This means it binds to the GABA binding site and prevents GABA from binding to it. GABA is an inhibitory neurotransmitter therefore arecaidine blocks the activity of GABA which leads to neural excitation. The hexane and aqueous fractions have also been shown to inhibit monoamine oxidase in rat brain homogenate favoring anti-depressant-like properties [12]. The main objective of this study was to investigate the anti-depressive property of the methanolic extract of *Areca catechu* nut on mice using the tail suspension test and the forced swim test.

Methodology

Sample collection

The Areca catechu nuts were collected from parklands subcounty in Nairobi (1.2599°S, 36.8179°E). A botanist confirmed the nut was the Areca catechu nut based on physical properties which were yellowish, red color with a conical shape with a flat base.

Preparation of areca catechu nut Methanolic Extract

Areca catechu nuts (5kg) were cut into small pieces and reduced to powder form using a blender at high speed. The powder was extracted using 450ml of Methanol in each beaker by maceration for 72 hours followed by concentration using rotary evaporator. The % yield was determined using the equation 1 below. From the extraction, 119.4g was extracted from a total of 5000g (5kg) of sample.

$$Yield = \frac{weight\ of\ of\ extract}{weight\ of\ sample\ used} X100 \text{ ----- ---------- (1)}$$

Equation 1. percent yield

Phytochemical Analysis

The *areca catechu* methanolic extract was tested for the presence of alkaloids, phenols, and tannins as described by Ahmady *et al*. [13]. Hager's test was used to test for alkaloids. Three drops of picric acid saturated solution were added to 2ml of the methanolic extract in a test tube. A yellow-whitish precipitate confirmed the presence of alkaloids. $FeCl_3$ test was used to test for phenols. Three drops of 1% $FeCl_3$ were dropped to 2ml of the methanolic extract. The presence of a greenish-black color confirmed the presence of phenolic compounds. To evaluate for the presence of tannins, gelatin assay was used. About 2ml of an aqueous solution with 1% gelatin and 10% NaCl were mixed with 2ml of the methanolic extract in a test tube. A whitish precipitate or a milky color confirmed the presence of tannins in the extract.

Acute Toxicity Studies

To ensure safety, an acute toxicity test was conducted in two phases, as described by Chinedu *et al* [14]. In phase one, 9 swiss albino mice were divided into 3 three groups each containing 3 mice. Three concentrations of 10, 100, and 1000 mg/Kg of Areca catechu nut methanolic extract were administered to the mice via oral gavage as shown in **Figure 1**. The mice were monitored every 5 minutes for behavioral and mortality for the first hour then monitored hourly for the next 24 hours. In phase two, 4 swiss albino mice were given different concentrations of the extract at 1600, 2900, 5000 mg/kg, and normal saline, respectively. Each group was monitored as described for phase 1. Lethal Dose 50 (LD_{50}) was computed according to equation 2 below.

$$LD_{50} = \sqrt{(C_1 X C_2)} \text{ ..... (2)}$$

C_1 = Highest dose with no mortality
C_2 = Lowest dose with mortality

Equation 2. Lethal dose 50

Figure 1. Oral gavage administration of *Areca catechu* methanolic extract into the mice.

Evaluation of Anti-Depressive Potential of *Areca catechu* Nut Methanolic Extract

A total of 50 mice were used for the antidepressant activity testing. The mice were divided into two groups of 25 mice each. One group for the tail suspension test and the other for the forced swim test. These two groups were further

divided into five groups of 5 mice each. The 5 groups were divided as follows:

Group one: Negative control (NC), administered saline 2 ml/kg/day orally.

Group two: Positive control (PC), administered standard drug Fluoxetine (10 mg/Kg/day orally).

Group three: Administered *Areca catechu* extract 50 mg/kg/day orally (AC50)

Group four: Administered *Areca catechu* extract 100 mg/kg/day orally (AC100)

Group five: Administered *Areca catechu* extract 200 mg/kg/day orally (AC200)

After administration via oral gavage, the mice were allowed to rest in their cages for 30 minutes before being evaluated using the forced swim test (FST) and tail suspension test (TST).

Forced Swim Test

Individual mice of either sex were made to swim in an open cylindrical container with a diameter of 10 cm and a height of 25 cm filled with water that was at 23.5°C and had a height of 19 cm. The extract, saline, or Fluoxetine treatments were administered 30 minutes before the study as described by Can, Dao, Arad, *et al*. [15]. All the mice were forced to swim for 6 minutes and the duration of immobility, climb time, and swim time were observed and measured during the last 4 minutes. The experiment was repeated for 10 days. When a mouse stopped wriggling and remained still in the water, moving only enough to keep its head above the water surface, it was deemed to be immobile. A decrease in the time spent immobile suggested an antidepressant-like effect [16].

Tail Suspension Test

This was done as described by Can, Dao, Terrillion, *et al*. [17] with a few modifications. Treatment with the extract, saline or fluoxetine was given 30 min preceding the study. With the aid of adhesive tape, mice were hung 50 cm above the ground on the edge of the table, 1 cm from the tip of the tail. The total duration of immobility induced by tail suspension was recorded over a 6 min period. The experiment was repeated for 10 days. Six minutes out of a total of ten minutes were used to record the entire amount of immobility caused by tail suspension. When the mice hanged passively and motionlessly without exhibiting any body movement, it was deemed to be immobile..

Results and Discussion

Percentage yield and Phytochemical Screening

From 5kg of the nut 119.4 g of dried methanolic extract was obtained which constituted 2.4% extraction yield. Preliminary phytochemical analysis of the methanol extract of *Areca catechu* showed the presence of alkaloids, phenols and tannins as shown in **Figure 2**.

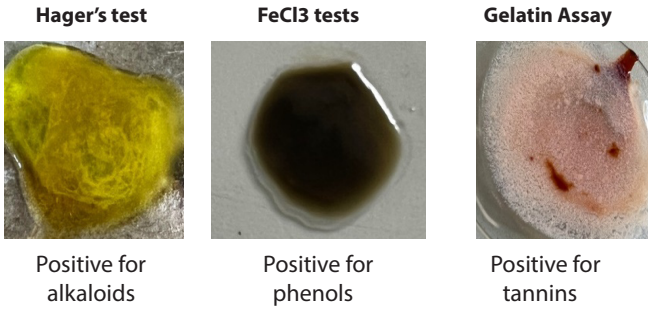

Figure 2. Phytochemical analysis of *Areca catechu* methanolic extract.

Presences of alkaloids such as arecoline, arecaidine, and guvacine as wells as phenols such as gallic acid have previously been reported in *Areca catechu* nuts and leaves [18]. Arecoline, arecaidine, guvacoline, and guvacine have been reported as *Areca nut*-specific alkaloids [19]. Arecoline is a pyridine alkaloid. The *Areca catechu* nut also has tannins present, the main ones being Catechins and epicatechins [20]. Hydrolysable tannins from *Terminalia catappa L*. have been reported to suppress stress induced depression through regulation of monoamine neurotransmitters levels and amelioration of oxidative stress [21]. Phenolic glycosides from the rhizomes of *Cyperus rotundus* have previousisly been shown to have antidepressant activity in mice using the forced swim test and tail suspension test [22].

Acute Toxicity Studies

All the mice in phase 1 (administered of low dose of 10mg/kg, 100mg/kg, and 1000mg/kg) survived. However, doses of 100mg/Kg and 1000mg/Kg showed to give the mice phases of erratic agitated behavior followed by calm sedentary behavior with palpitations resolving after a few minutes. The dose dependent effect on mice showed that toxicity would increase at higher doses. The toxic effects were seen in phase 2 where doses of 1200mg/kg to 5000mg/kg were used. All mice in this phase died apart from the mice administered with normal saline. From this data, the LD-50, which is the dose at which 50% of the animals died, was calculated to be 1265mg/Kg.

Antidepressant activity

The measure of the duration of immobility in Forced swim test and Tail suspension test was used to assess the antidepressant effects of the methanolic extract of the nuts. The average mobility times of the mice administered with different doses is shown in **Table 1** while change in body weight is shown in **Figures 3 and Figure 4**. There was notable difference in mobility for control mice compared with treated mice while notable weight loss in both models was observed over the study period. The physical appearance of the fur of mice over the treatment period is shown in **Figure 5**. The untreated mice had rough fur.

Table 1. Effect of *Areca catechu* nuts on immobility time in Forced swim test and Tail Suspension test.

Treatment	Dose (mg/Kg/ day)	Forced Swim test Duration of Immobility (Sec)	Tail Suspension test Duration of Immobility (Sec)
Methanolic extract	50	80± 34	26± 2
Methanolic extract	100	77± 19	57± 9
Methanolic extract	200	101± 26	100± 20
Normal saline	Negative control (normal saline)	51± 12	23± 3
Fluoxetine	Positive control (Fluoxetine)	96± 24	103± 5

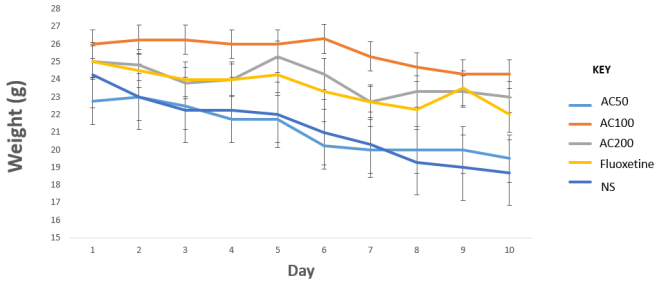

Figure 3. Average weight of 5 groups of mice during the 10-day forced swim test following administration of different concertation of the *Areca catechu* methanolic extract. AC50:50 mg/kg/day; AC100: 100 mg/kg; AC200: 200 mg/kg; NS, Normal Saline.

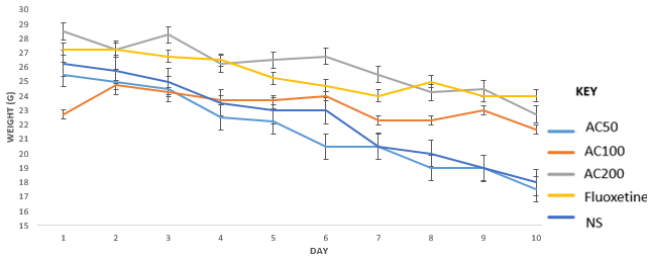

Figure 4. Average weights of 5 groups of mice over 10 days' tail suspension test following administration of different concertation of the *Areca catechu* methanolic extract. AC5050 mg/kg/day; AC100: 100 mg/kg; AC200: 200 mg/kg; NS, Normal Saline.

Figure 5. Appearance of mice receiving different doses of *Areca catechu* nut methanolic extract. From left to right- negative control (NS), lowest dose (50mg/Kg), highest dose (200mg/Kg).

The group that was given the methanolic extract of 200 mg/kg of mice displayed the highest average swim time of 101±26 sec. This was close to the positive control group which had fluoxetine which showed an average swim time of 96± 24. This finding indicates the potential comparable pharmacologic activity of the methanol extract of *Areca catechu* and fluoxetine, which could be due to inhibition of the reuptake of serotonin. The methanolic extract at concentrations of 50mg/kg and 100mg/kg showed similar average swim times at 80±34 seconds and 77±19 seconds, respectively. These averages were still higher than the negative control group (normal saline) which had an average of 51±12 seconds.

A dose dependent effect was seen for the average mobility time during the tail suspension test suggesting the higher the dose, the higher the mobility time. The highest mobility time was recorded for the group administered with the positive control (fluoxetine) at 103±5 seconds which was comparable to the group administered with 200 mg/kg of mice, which had an average mobility time of 100±20 seconds. The group that was given 100 mg/kg of mice had an average mobility time of 57±9 seconds. The average mobility time of this group was still higher than the negative control but lower than the highest dose and positive control indicating minimum anti-depressive activity. The lowest average mobility time was recorded from the negative control group administered with normal saline at 23±3 which was comparable to the average mobility time of the 50mg/kg group, which had an average mobility time of 25.88±2 seconds. This indicates the lowest concentration during the tail suspension test showed no anti-depressive activity.

Antidepressant effect of various plant alkaloids has been reported in the literature [23]. Pyridine alkaloids derived from plant bases have shown to have an effect on the central nervous system [24]. Arecoline specifically partially, non-selectively binds to muscarinic and nicotinic acetylcholine receptors. This increases acetylcholine levels. Acetylcholine reduces heart rate and dilates vessels. This effect of acetylcholine aids in anxiety. Arecoline easily crosses the blood brain barrier [25] in order to cross the blood brain barrier, a compound is supposed to be polar and small in size below 600Da. A similar study done by Babu [26] showed the extract of *Dacus Carota* had anti-depressive activity as the immobility time of the mice reduced as the extract was seen to increase the motor activity of mice which reduced the depressive symptoms. Antidepressant activity of the extract was noted by the reduction in immobility in both tests, which could be indicative of the potential efficacy of the extracts in reliving depression.

A decline in mice weight over the ten days of drug administration was observed in both treatments. One of the symptoms of depression is lack of appetite [27].The decline in weight in the groups with a high average swim time (200mg/kg, fluoxetine, 50mg/kg, and 100mg/kg) can be explained by an increase in serotonin levels. As serotonin levels rise, the level of satiety reduces [28]. A study done by

Uguz *et al*. [29] shows a link between short-term use of selective serotonin reuptake inhibitors and weight loss. The average weight of all groups of mice during the TST decreased over the 10-day period as seen in **Figure 4**. The average weight of the mice administered the extract of 50mg/Kg and negative control showed the highest decrease in weight. This could be attributed to little or no anti-depressive activity shown by normal saline and the *Areca catechu nut* methanolic extract at a concentration of 50mg/kg. This is seen in **Table 1** with the average mobility time of the negative control (NS) and AC50 groups being the lowest. The average weights of the other groups declined. This could be due to satiety levels varying because of the extract and the test carried out on the mice. Other factors may also have contributed to the weight loss like feeding habits and competition in the cages for feeding.

An observation made during the study showed the appearance of the fur of the mice varied with mice in negative control group having ruffled fur and normal looking fur in the mouse in the 200Mg/Kg group. According to Quesenberry [30] ruffled fur is a sign of illness. In the FST, the negative control mice were made to experience depression, which was not alleviated by the administration of the extract or medication. The forced swim test that the mice in Figure 5 underwent may have caused a depression that led to their fur to become ruffled.

Study Limitations

In this study, the mice were housed in only two separate cages separating the group of the tail suspension test from the group of the forced swim test. This meant that each cage had 25 mice each which would increase competition for food, water, and warmth. This would affect the general mood of the mice to a small extent. This would influence the results during the forced swim test and tail suspension test

Conclusion

Areca nut methanolic extract showed anti-depressive activity at different concentrations. The antidepressant activity was dose dependent with best anti-depressive activity being observed at the highest concentration used in this study. Mice at this concentration had the highest swim time and mobility time, respectively which confirmed the anti-depressive activity. The antidepressive effects of the groups receiving 200 mg/Kg of the methanolic extract of *Areca nut* were comparable to the positive controls (fluoxetine).

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Epipremnum aureum (Linden and André) G. S. Bunting (Araceae): Free Radicals Scavenging, Cell Growth Inhibition and Cytotoxic effects on Rhabdomyosarcoma and Laryngeal Carcinoma Cancer Cells

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Abstract

Patients with chronic health conditions such as cancer often benefit from plant based herbal recipes with minimal adverse effects. There also exists growing worries over the safety and efficacy of most of the currently used conventional chemotherapeutic agents. This study examined the radical scavenging and cytotoxicity of *E. aureum* traditionally employed for the management of cancerous diseases.

The aerial part of *Epipremnum aureum* was collected from the University of Ibadan botanical garden and authenticated at the Forest Herbarium Ibadan. Air-dried aerial part of *Epipremnum aureum* was pulverized and extracted with 70% aq. methanol and concentrated in vacuo. A portion the methanol extract was fractionated and crude alkaloidal extract was prepared from another portion of the methanol extract. The extracts and fractions were subjected to; antioxidant DPPH radical scavenging assay and cytotoxicity of the extracts and fractions against rhabdomyosarcoma (Rd) and laryngeal carcinoma (Hep-2C) cancer and non-cancerous (Vero) cell lines were determined.

Ethyl acetate fraction and alkaloidal extract displayed better DPPH radical scavenging activity than the methanol extract and other fractions. The alkaloidal extract (CC50=0.17±0.03µg/mL) and dichloromethane fraction (CC50=0.28±0.05 µg/mL) displayed the highest cytotoxicity against Rd cancer cell lines compared to cyclophosphamide (CC50=2.23±0.33 µg/mL). However, the alkaloidal extract displayed the least cytotoxicity to Vero cell line and it is about 14.25 and 22.18 times more cytotoxic to Rd and Hep-2C cancer cells, respectively.

This present study demonstrated that *E. aureum* possess antioxidant and cytotoxic activities with less cytotoxicity on healthy cells.

Key words: *Radical Scavenging, Cytotoxicity, GC-MS Analysis, Epipremnum aureum.*

Introduction

Cancer remains a leading cause of death and about 19.3 million new cancer cases and 10.0 million cancer related deaths were reported recently in the year 2020 alone (1). The emergence of chemoresistant cancers is prosing new challenges to cancer chemotherapy with many anticancer drugs become ineffective for management of metastasised cancers (2). The narratives of anticancer drug discovery is tightly woven with medicinal plants and many chemotherapeutic drugs including paclitaxel, vincristine, podophyllotoxin own their origin to medicinal plants (3). Cancer such as rhabdomyosarcoma and laryngeal carcinoma has been reported to sometimes arise from activity of free radicals generated from sources such as tobacco smoke and alcohol (4). Rhabdomyosarcoma type of cancer arises from soft tissues of the skeletal muscle tissue or hollow organs such as the bladder and uterus (5). While, laryngeal carcinoma is form in the tissues of the larynx and like the rhabdomyosarcoma are influenced by environmental and lifestyle-related factors, such as tobacco use, consumption of alcohol, and exposure to toxic substances (6). Antioxidants have been reported to reduced the risk of some forms of cancer, including skeletal muscle, larynx, prostate, colon and breast cancer risk (7). The process of new anticancer drug discovery from medicinal plants often commence with *in vitro* cytotoxicity screening of medicinal plants followed by isolation of anticancer compounds from active plants (8).

Epipremnum aureum (Linden and André) G.S. Bunting, belongs to araceae family (9) is an evergreen climber (10, 11). It is commonly grown indoors where is used for beautification and purification of polluted air (12-14).

Epipremnum aureum possess antibacterial, anti-termite and antioxidant properties and traditionally in Malaysia and Singapore it is popularly used as a major ingredient in the preparation of traditional anticancer remedy (15, 16). The aim of this study was to assess the radical scavenging and anticancer potential of *E. aureum* aerial part and identify phyto-constituent present in the plant.

Methodology

Plant Material

The aerial part of *Epipremnum aureum* was collected from the University of Ibadan botanical garden and authenticated at the Forest Herbarium Ibadan (FHI 112371).

Preparation of extracts and fractions: Air-dried *E. aureus* (2000g) powder was extracted exhaustively into 70% aqueous methanol (3L). The extract was concentrated in vacuo at 45˚C, labelled as crude extract and stored in refrigerator at 4˚C until it was needed. Fifty grams (50g) of crude extract obtained above was partitioned into n-hexane, dichloromethane (DCM), ethyl acetate (EtoAc) and butanol soluble fractions. All fractions obtained were concentrated in vacuo at 45˚C and stored in the refrigerator at 4˚C until they were needed.

Alkaloidal Extraction: One hundred gram (100 g) of crude extract obtained above was dissolved in 10% HCl solution (500 mL) and defatted with 500 mL each of n-hexane (5X) to remove chlorophyll, lipids and other non-polar constituents. The aqueous solution was made basic to pH 10 with dilute NH4OH and extracted (4 X) each with chloroform (500 mL) as shown in Figure 1. The combined chloroform phase was concentrated and yielded 12 g of crude alkaloid extract which was stored in the refrigerator (4 oC) until further use (17). The percentage yield of extract and fraction (% w/w) was calculated using the formula below;

$$\%\ yield\ of\ extract = \frac{weight\ of\ of\ extract}{weight\ of\ spowdered\ plant\ material} X100\%$$

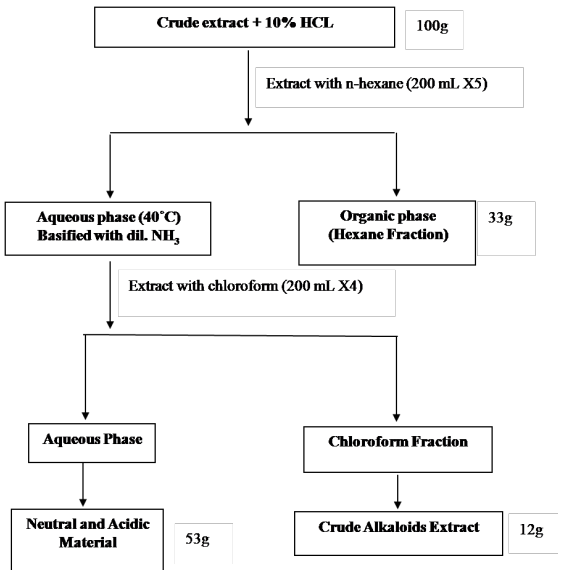

Figure 1. Extraction of Crude Alkaloids from E. aureum Crude Extract.

1, 1-diphenyl-2-picryl hydrazyl (DPPH) scavenging activity

The DPPH radicals scavenging ability of the extracts and fractions was assessed by a method previously reported (18). From a stock solution (1000 mg/mL) of the extracts and fractions, serial dilutions of 200, 100, 50, 25, 12.5 and 6.25 µg/mL were prepared in the 96 well-plate and 150 µL of 0.04 µg/mL DPPH in methanol was added to each well and the microplate plate was incubated for 30 minutes in the darkness at room temperature (25 ˚C). The UV absorbance (Absorbance sample) of the resulting solution was measured at 517 nm using the Ultra-violet spectrophotometer (Titertek Uniskan). Control simple contained DPPH and solvent (methanol) without the extract. Ascorbic acid was used as positive controls while methanol as negative control. This experiment was carried out in triplicate. The results obtained were expressed as percentage inhibition and calculated by the formula below.

$$Inhibition\ (\%) = \frac{Absorbance\ control - Absorbance\ sample}{Absorbance\ control} X100\%$$

Growth inhibitory activity

The extracts and fractions were subjected to growth inhibitory assays as described below;

Sorghum bicolor radicle growth inhibitory assay: Fresh seeds of *Sorghum bicolor* were purchased from *Ipata* market in Ilorin. Viable seeds were selected by introducing a 100 g of the seeds in distilled water. The floating seeds and husk were decanting off while the submerged seeds were collected aired and washed with 95% ethanol for a minute and later rinsed with distilled water before they were dried and stored in dried dark place until needed. Only viable seeds were used for *Sorghum bicolor* radicle growth (SBRG) inhibitory assay. Ten milliliters of 39.06, 156.25, 625, 2500 and 10000 µg/mL were prepared by a fourfold dilution of the stock solution (40 mg/mL) prepared in 5% DMSO. Cyclophosphamide was used as the positive control at similar concentration with the extract/fractions. A volume of 10 mL different concentrations of the extracts (39.06 - 10000 µg/mL) were transferred into pre-labelled petri-dish of 10 cm lined with filter paper underlie with wool, thereafter ten (10) seeds were placed on each filter paper in the petri-dishes. The petri-dishes were incubated in a dark cupboard at ambient temperature (32˚C) and the lengths of emerging radicle were measured after 48 and 96 h of incubation. Seeds of the negative control group were treated with 10mL of 5% DMSO in distilled water. This experiment was performed in triplicates. With the aid of a scaled ruler changes in radicle length were measured. The mean change in radicle length per concentration was calculated. The percentage radicle growth inhibition at 48 and 96 h and IC_{50} were determined (19).

Percentage inhibition of the root growth was calculated using formula below; (next page)

$$inhibition\ (\%) = \frac{A\_B}{A}\ X100\%$$

Where; A = length of untreated root.
B = length of treated root with plant extracts/ cyclophosphamide

***Allium cepa* root growth inhibitory assay:** *Allium cepa* root growth (ACRG) inhibitory assay was conducted using method described in our earlier work (19). Moderate size onion bulbs (50±10 g) were obtained from *Ipata* market. Allium cepa bulbs (50±10 g) were rinsed with water (distilled) and grown with the root submerged in water in the dark at room temperature (32˚C) for 24-48 h until the roots have grown up to 3cm length.

Twenty (20) millilitre of different concentrations (39.06, 156.25, 625, 2500 and 10000µg/mL) of each extract was prepared by four fold dilution of the stock solution (40mg/mL) prepared in 5% DMSO. Twenty (20mL) of various concentration were transferred into the petri-dish of 10cm diameter and the roots of each of onion bulbs submerged in the extract/fraction in the petri-dishes containing each extract/fraction (39.06-10000 µg/mL). Cyclophosphamide was used as the positive control at similar concentration with the extract/fractions, while 5% DMSO in water was applied as the negative control. This experiment was performed in triplicates. New root growth lengths were measured at 0, 48, 96h and the percentage root growth inhibition were estimated by the formula below;

Percentage inhibition was calculated as:

$$inhibition\ (\%) = \frac{A\_B}{A}\ X100\%$$

Where; A = length of untreated radicle.
B = length of treated radicle with plant extracts/ cyclophosphamide

Cytotoxicity assays

Cytotoxic potential of the extracts and fraction of *E. aureum* were determined by the cytotoxic potential of the test samples on the nauphill of Artemia salina and malignant cell lines as described below.

Brine shrimp lethality (BSL) assay: The eggs of *Artemia salina* were incubation in seawater for 48 h to hatch the eggs. Ten milligrams of the extract was dissolved in 2mL 5% DMSO in sea water and resulted in 5mg/mL stock solution. Further dilutions of 1, 10, 100, 500 and 1000 µg/mL were made in microplates in triplicates. A 250 µL suspension of nauplii in the extract was added to each well. The plates were incubated at room temperature (RT = 25-33°C) for 24 h. 0.5% DMSO in sea water was used as the negative control and thereafter the number of dead nauplii in each well was counted(20).

The percentage cytotoxicity was estimated by the formula below;

$$\%\ inhibition\ (\%) = \frac{A\_B}{A}\ X100\%$$

Where; A = the mean number of shrimp in negative control wells.
B = the mean number of shrimp in the wells with plant extracts/cyclophosphamide.

Extract(s) or fraction(s) with LC_{50} values <100µg/mL are regarded as active and are selected for further cytotoxicity studies. The method was only employed for preliminary screening fractions and compounds obtained were not tested using this model. Extracts that demonstrate strong brine shrimp lethality are regarded as promising candidate for anticancer drug discovery.

Cytotoxicity of extracts and fractions on Rd and Hep-2C cancer cell lines: Cytotoxicity of the extracts and fractions were determined by MTT (3-(4, 5-dimethyl thiazole-2yl)-2, 5-diphenyl tetrazolium bromide) assay using the method described by (21). Solutions of extracts for MTT assay were prepared by dissolving 10 mg of extract/fraction in 1 mL of DMSO separately to give a concentration of 10mg/mL. Stock (0.1mL) solution prepared above was added to 0.9 mL of medium to obtain a dilution of 1000µg/mL, there after ten-fold serial dilutions of the samples were prepared from stock solution to obtain graded concentrations (0.01, 0.1, 1, 10, 100 and 1000 µg/mL). Thereafter, 50µL of each sample solution was dispensed into 96-well microtitre plates already seeded with monolayer of cells in triplicates. The plates were incubated at 37˚C in a carbon-dioxide environment and the cells observed under microscope after 72 h. Supernatants were removed from all wells after 72h incubation at 37˚C and 25µL of 2 mg/mL MTT (sigma) solution in phosphate buffer saline (PBS) was added to each well and the plates were incubated for 2h at 37˚C. Thereafter, 125µL of DMSO was added to the wells to dissolve the MTT crystals formed. The UV absorbance of resulting solution was measured using microplate reader (Titertek Uniskan) at wavelength of 570nm against a background control as blank. The UV absorbance of each plate directly correlates to the number of viable cells in each well.

Percentage inhibition of cancer cell proliferation was calculated by the formula below;

$$\%\ inhibition\ (\%) = \frac{A\_B}{A}\ X100\%$$

Where; A = the mean optical density of untreated cells.
B = the optical density of cells treated with plant extracts/fractions (22)

The IC_{50} was determined from a non-linear regression curve plotted using Graphpad prism version 6.0

The selective index (SI) of the two extracts and cyclophosphamide were estimated using the Vero cells. Data were expressed as the means ± SEM. When SI is greater than 1, it is indicative of selectivity (20).

$$\text{Selective index (SI)} = \frac{CC_{50}\text{ on Vero cells}}{CC_{50}\text{ on cancer cells}}$$

Phytochemical analysis

Preliminary phytochemical screening: The extracts and fractions of *E. aureum* were screened for the presence of

secondary plant metabolites as described below using the method previously reported (23).

Gas chromatography mass spectrometry (GC-MS) analysis: The phytochemical investigation of the n-hexane fraction of *E. aureum* was performed using a GC-MS (Thermo Scientific Co.) equipment. The GC-MS instrument controlled parameters are as follows: Injection source (PAL Sampler), injection volume (2µL), column (Agilent technologies-HP-5MS), column dimension (360˚C: 30mx250µm x 0.25µm), initial temperature (50˚C), program temperature (260˚C), initial pressure (9.05psi), average velocity (38.724cm/sec), holdup time (1.2912min), run time (82.286min). Sample dissolved in HPLC grade hexane was run full and compared by using Database- Spectrum MS-NW-1798. Suggestions with the highest confidence interval greater than 80% were selected. Compounds were identified based on comparison of their retention and mass spectra data to those generated under identical experimental conditions by applying a two-dimensional search algorithm, considering the retention index, as well as mass spectral similarity.

Statistical analysis

Data obtained were expressed as means ± SEM of values obtained in triplicates from three independent experiments. Statistical differences between treated groups and standards were evaluated using one way analysis of variance (ANOVA). A p value <0.05 was considered to be significantly. Data were analysed using GraphPad Prism Version 6.0 Software.

Results

Extracts preparation and radical scavenging activity: The plant material yielded 10.78% w/w of dark green extract. Among the fractions obtained, n-hexane (2.96%$^{w}/_{w}$) had the highest yield, while dichloromethane (0.34 %$^{w}/_{w}$) had the lowest yield. Alkaloid extract (0.12g) was obtained per 1g of the crude extract as shown in the **Table 1** below. The ethyl acetate fraction (109±4.04µg/mL) and alkaloidal extract (57.43±3.33µg/mL) displayed better DPPH radical scavenging activity than the crude extract (IC_{50} of 157.06±3.62µg/mL) and other fractions as shown in **Table 1**.

Growth inhibitory and cytotoxic activities: The crude extracts (GI_{50} of 60.00±2.73 and 356.00±6.24µg/mL) displayed growth inhibitory effect in SBRG and ACRG inhibitory assays. The alkaloidal extract (GI_{50}=45±3.26 and 215±9.92µg/mL) demonstrated better growth inhibitory effects to cyclophosphamide (GI_{50}=705.33±11.69 and 151.50±0.52µg/mL) in SBRG and ACRG inhibitory models while the other fraction all weaker growth inhibitory effects (**Table 1**). Similarly, the alkaloids extract (LC_{50}=82±4.26 µg/mL) and dichloromethane fraction (LC_{50}=99±5.57µg/mL) displayed cytotoxic effect on the nauplii of Artemia salinathat was comparable to cyclophosphamide (LC_{50} of 98±3.33µg/mL) and better than the methanol extract and other fractions (**Table 1**).

Interestingly, alkaloids extract (CC_{50}=0.17±0.03 and 0.21±0.03 µg/mL) and dichloromethane fraction (CC_{50}=0.28±0.05 and 0.43±0.06µg/mL) displayed better cytotoxic effects cyclophosphamide (CC_{50}=2.23±0.33 and 2.66±0.78 µg/mL), methanol extract (CC_{50} of 2.92±1.06 and 2.51±1.80 µg/mL) and other fractions against Rd and Hep-2C cancer cell lines (**Table 1**). However, the crude extract (10±1.76 µg/mL), dichloromethane fraction (2.99±0.33µg/mL) and alkaloidal extract (2.44±0.54µg/mL) all displayed lesser cytotoxicity on the normal non-cancerous (Vero) cells and the estimated selectivity index of the crude extract (3.43 and 3.98), dichloromethane fraction (10.68 and 6.95) and alkaloids extract (14.35 and 22.18) for Rd and Hep-2C cancer cell lines (**Table 1**).

Phytochemical constituents: Preliminary phytochemical analysis of the extracts and fractions revealed the presences of alkaloids, flavonoids, tannins, saponins, anthraquinones and steroidal terpenoids (**Table 2**). However, the GC-MS analysis of the n-hexane fraction led to identification of 49 bioactive compounds (**Figure 2 and Table 3**). The most abundant constituents of the fraction included; phytol (21.51% occurrence), toluene (21.44% occurrence) and n-Hexadecanoic acid (16.17% occurrence). Other compounds include; hexadecanoic acid, methyl ester, 9,12-Octadecadienoic acid and 9,12,15-Octadecatrienoic acid.

Table 1. Yield (%$^{w}/_{w}$), antioxidant, growth inhibitory and cytotoxic activities of *E. aureus* Extracts and Fractions.

Samples	DPPH Radical scavenging IC50±SEM (µg/mL)	DPPH Radical scavenging IC50±SEM (µg/mL)	Growth inhibition (µg/mL)		Cytotoxicity (µg/mL)				Cancer Cell Selective Index (SI	
			SBRGI GI50±SE¬M	ACRGI GI50±SEM	BSL LC50±SEM	Rd Cell Line CC50±SEM	Hep-2c Cell Line CC50±SEM	Vero Cell Line+++ CC50±SEM	Rd Cell Line	Hep-2C Cell Line
Crude Extract	10.78	157.06±3.62*	60.00±2.73*	356.00±6.24*	200±2.16*	2.92±1.06	2.51±1.80	10±1.76	3.43	3.98
n-Hexane	2.96	> 400*	620.80±42.41	2156.00±77.05*	453±42.73*	6.98±2.65*	1.00±0.54*	1.24±0.67*	0.18	1.24
Dichloromethane	0.34	320±19.74*	1126.40±37.96*	1571.00±17.01*	99±5.57	0.28±0.05*	0.43±0.06*	2.99±0.33	10.68	6.95
Ethyl Acetate	0.72	109±4.04*	193.90±9.20*	310.70±13.89*	250±8.69*	11.19±0.96*	1.94±0.67	0.67±.59*	0.06	0.35
Butanol	1	348±16.48*	1412.64.±53.95*	2388±0.13*	633±33.53*	7.77±1.33*	2.63±0.33	0.38±0.04*	0.05	0.14
Aqueous	0.83	> 400*	1078±31.73*	4501.00±61.60*	658±15.62*	5.18±8.69*	4.92±0.56*	0.22±0.09*	0.04	0.04
Crude Alkaloids	0.12	57.43±3.33*	45±3.26	215±9.92	82±4.26	0.17±0.03	0.21±0.03	2.44±0.54	14.35	22.18
Standard Drug		9.26±0.00+	705.33±11.69++	151.50±0.52++	98±3.33++	2.23±0.33++	2.66±0.78++	8.71±0.56++	3.91++	3.27++

Key:
SBRGI = Sorghum bicolor radicle growth inhibition: ACRGI= Allium cepa root growth inhibition: *=Significantly Different from Standard Drug (P<0.05): + = IC_{50} of Ascorbic acid: ++ = IC_{50} of Cyclophosphamide: +++ = Normal non-cancerous cell line.

Table 2. Phytochemical Examination of Selected Plant Extract.

Bioactive constituent	Chemical Test	Crude Extract	n-hexane fraction	Dichloro-methane fraction	Ethyl acetate fraction	Butanol fraction	Aqueous fraction	Alkaloid extract
Alkaloid	Drangendorff's	++	-	++	-	-	-	+++
	Wagner's	++	-	++	-	-	-	+++
	Meyer's	++	-	++	-	-	-	+++
Flavonoid	Shinoda's test	++	++	+	+++	++	++	-
	Lead ace tate	+	-	+	+++	++	++	-
Tannins	Fecl3	+	-	+	+	++	+++	-
Saponins	Frothing	+++	-	-	+	++	+++	-
	Emulsifying	++	-	-		++	+++	-
Anthraquinone	Combined	++	-	-	-	+	++	-
	Free	+		-	-	+	++	-
Cardiac glycoside	Keller killiani	-	-	-	-	-	-	-
	Kedde	-		-	-	-	-	-
Steroidal terpenes	Salkowski	++	+++	+	-	-	-	+

Table 3. Chemical composition of the hexane fraction of E. aureum aerial part

No	Compounds	RT	% Occurrence
1	Toluene	6.46	21.44
2	Ethanone	19.79	0.02
3	Decanoic acid	22.5	0.02
4	2,5,5,6,8a-Pentamethyl-trans-4a,5,6,7,8,8a-hexahydro-gamma-chromene	24.08	0.02
5	Dodecanoic acid	25.16	0.08
6	Dihydroactinidiolide	25.21	0.05
7	Fumaric acid, ethyl 2-propylphenyl ester	25.46	0.11
8	Tricyclo[4.3.1.1(3,8)]undecane, 1-methoxy-	26.58	0.03
9	1-(2,6,6-Trimethyl-2-cyclohexen-1-yl)acetone	27.43	0.03
10	Methyl 12-methyl-tridecanoate	27.81	0.06
11	Tetradecanoic acid	28.47	0.31
12	Thiazolo[4,5-b]pyridin-2(3H)-one, 5-hydroxy-7-methyl-6-propyl-	28.92	0.22
13	Pentadecanoic acid, methyl ester	29.1	0.05
14	Methyl 12-methyltetradecanoate	29.3	0.1
15	Pentadecanoic acid	29.87	0.11
16	Pentadecanoic acid, methyl ester	29.95	0.14
17	Propyl 12-methyltetradecanoate	30.11	0.25
18	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	30.36	0.37
19	2-Pentadecanone, 6,10,14-trimethyl-	30.52	0.24
20	Pentadecanoic acid	30.83	0.59
21	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	31.01	0.04
22	Phthalic acid, butyl tetradecyl ester	31.32	0.09
23	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	31.54	0.1
24	Hexadecanoic acid, methyl ester	31.67	0.07
25	Hexadecanoic acid, methyl ester	32.86	8.36
26	n-Hexadecanoic acid	34.59	16.17
27	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	40.47	4.8
28	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	40.89	5.04
29	Phytol	41.73	21.51
30	Heptadecanoic acid, 16-methyl-, methyl ester	42.33	2.12
31	9,12-Octadecadienoic acid (Z,Z)-	43.71	5.87
32	Linolenic acid	44.2	3.46
33	Stearic acid	45.11	0.43
34	Eicosanoic acid, methyl ester	50.97	0.87
35	cis-Vaccenic acid	51.61	0.21
36	(2,2-Dimethyl-6-methylene-cyclohexyl)-3-methyl-pent-2-enyl]-[1,4]benzoquinone	52.35	0.16
37	Methyl 11-(3-pentyl-2-oxiranyl)undecanoate	53.27	0.04
38	Methyl 14-methyl-eicosanoate	55.12	0.3
39	Benzenedicarboxylic acid, diisooctyl ester	55.47	0.84
40	Methyl-9-tetradecen-1-ol acetate	57.63	0.05
41	Oleic acid	57.76	0.03
42	Oleic acid	58.73	0.09
43	Heptatriacotanol	59.27	0.04
44	Trimethyl-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetraenyl)-cyclohexanol	59.38	0.51
45	γ-Tocopherol	62.8	0.2
46	α-Tocopherol	64.26	0.72
47	Stigmasterol	67.29	0.76
48	γ-Sitosterol	68.71	0.51
49	ß-Sitosterol	71.48	1.27

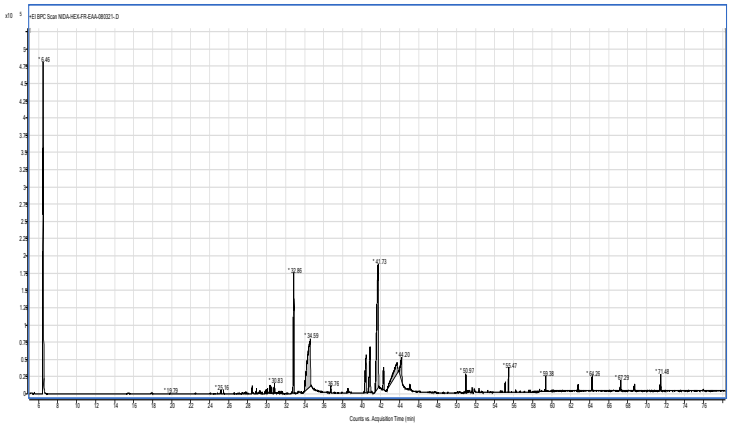

Figure 2. GC-MS Chromatogram of the hexane fraction of E. aureum aerial part.

Discussion

About 80% of the global population utilize medicine that are derived or inspired from plants (24). It is reported that patients with chronic health conditions such as diabetes, hypertension and cancer often benefit tremendously from plant based herbal recipes and they often experience minimal adverse effects (25), however because of the growing worries over the safety and efficacy of most of the currently used chemotherapeutic agents. There is need to explore natural products for identification of new leads with cytotoxic and antioxidant potential. This study examined the radical scavenging, growth inhibitory and cytotoxic potential of *E. aureum* extracts with a focus to identify the class of phytochemical present in the plant and identify compounds present in the plant.

The crude extract displayed some radical scavenging, growth inhibitory and cytotoxic activities. However, the fractions and alkaloids extract displayed different activities across the different test models. The n-hexane fraction was found to be rich in steroidal terpenes and flavonoids and the apolar fraction was most active against Hep-2C cancer cells (1.00±0.54µg/mL) and the GC-MS analysis of the fraction lead to identification of 49 compounds and the most abundant constitutes include; phytol, n-hexadecanoic acid,hexadecanoic acid, 9,12-octadecadienoic acid and

9,12,15-Octadecatrienoic acid. Other important bioactive compounds identified in the fraction include; dihydroactinidiolide, Linolenic acid, Vaccenic acid (an omega-7 fatty acid), Oleic acid, (γ & α-Tocopherols), Stigmasterol and sitosterol (γ & ß-Sitosterol). Interestingly, phytol has been reported to demonstration potent cytotoxic effect against lungs cancer cell by induces apoptosis in A549 cells by depolarizing the mitochondrial membrane potential(26). Similarly n-hexadecanoic has been reported to posses some antiprolifarative activities (27). Dihydroactinidiolide is a powerful anti-proliferative compound that had been reported to regulates Nrf2/HO-1 expression and inhibits caspase-3/Bax pathway to protect SH-SY5Y human neuroblastoma cells from oxidative stress induced neuronal apoptosis (28). Other notable compounds with antiproliferative activities include both γ & α-Tocopherols. Tocopherols have been reported to possess cytotoxic effect on prostate cancer cell lines (29). Stigmasterol and sitosterol have been reported to possess cytotoxic effects on MCF-7 breast cancer cell lines (30).

On the other hand the dichloromethane fraction and alkaloidal extract contain similar chemical contents and the displayed similar cytotoxicity against nauplii of *Artemia salina* and cancer cell lines. This may infer that the alkaloidal content of the dichloromethane fraction and those present in the alkaloidal extract maybe responsible for the observed cytotoxicity. Interesting earlier report of the GC-MS analysis of the alkaloidal extract of *E. aureum* led to identification of twenty six structurally different alkaloids among which includes known cytotoxic alkaloids such as; Catharanthine that is clinically used in the treatment of Hodgkin's lymphoma, nonsmall-cell lung, bladder, brain, and testicular cancers (17, 31).

Conclusion

This present study demonstrated that *E. aureum* possess antioxidant and cytotoxic activities and the plant contain alkaloids that display potent cytotoxic activities against human rhabdomyosarcoma and laryngeal carcinoma. It is recommended that the cytotoxic activity of the plant should be examined in vivo and the plant extract should be explored for the isolation of the anticancer compounds.

Conflict of interest

There is no conflict of interest with the data contained in this manuscript.

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Cost-effectiveness of Alternative Strategies to Initiate Antiretroviral Therapy in Iran: A model-based analysis

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Abstract

Background: Iran, along with 18 other nations, constitutes the Middle East and North Africa (MENA) region. This region has experienced an increase in the number of new HIV/AIDS cases and AIDS-related deaths between 2010 and 2019 despite decreasing global trends.

Objective: This study aimed at evaluating the cost-effectiveness of alternative strategies for initiating antiretroviral therapy in Iran.

Methods: Decision-analytic tree and Markov model were used to evaluate the clinical and economic consequences of the new WHO recommendations for the initiation of antiretroviral therapy. The two arms of initiation evaluated were CD4 < 350 and > 500 cells/ml. Disability-adjusted life years (DALY) averted, percentage DALY averted, Incremental cost and Incremental cost-effectiveness ratio (ICER) were determined. Input parameters were hazard rates of events, costs at health payer's perspective, annual healthcare cost for HIV from Imam Khomeini hospital Tehran. Deterministic and Probabilistic sensitivity analysis (Monte Carlo) simulations were then executed.

Results: Annual healthcare cost for HIV was $599.25 per prevalent case; immediate Antiretroviral therapy arm (ART) caused 0.30 (115%) DALY aversion. Incremental costs were -$303.29 while ICER was -$978.35. This ICER was robust to deterministic and probabilistic sensitivity analysis.

Conclusion: Starting ART in patients with CD4 count > 500 cells/mL was cost-effective in managing HIV in Iran.

Key words: *Antiretroviral therapy, Cost-effectiveness Analysis, Human immunodeficiency virus, Acquired Immune Deficiency Syndrome, Iran.*

Introduction

Acquired Immune Deficiency Syndrome (AIDS) / Human Immunodeficiency virus (HIV) are diseases that causes a defect in cellular mediated immunity. They were first mentioned in the Centers for Disease Control's Morbidity and Mortality Weekly Report in 1982. Globally, there were around 36.7 (34.0-39.8) million people living with HIV (PLWH) at the end of 2015 (1).

Antiretroviral therapy (ART) is the main therapy for the management of HIV. Although different methods were used in previous years, the World Health Organization (WHO) initially recommended that HIV ART be initiated only for patients at the highest risk of morbidity and mortality, the majority of whom have low CD4 cell levels (2). Three significant randomized controlled trials, Strategic Timing of Antiretroviral Treatment (START), TEMPRANO, and HIV Prevention Trials Network (HPTN 052), have recently shown the clinical benefit of early antiretroviral therapy initiation/ initiation in patients with high CD4 counts (3). The outcome of these clinical trials led the WHO to gradually increase the CD4 count initiation thresholds. As such, by 2015, recommendations were made for therapy to be administered instantly to all patients irrespective of CD4 levels (3).

Iran, along with 18 other nations, constitutes the Middle East and North Africa (MENA) region. This region has experienced an increase in the number of new HIV/AIDS cases and AIDS-related deaths between 2010 and 2019 despite decreasing global trends (4). Although the disease has a low prevalence (0.1%) in the general population, it has a high prevalence among the most at-risk populations (MARPs (5). This category of epidemic in Iran (where prevalence is > 5% among certain populations), referred to as a "concentrated epidemic," if ignored and not combated by effective measures, has the risk of developing into a widespread epidemic (6).

The fourth national strategic plan for HIV/AIDS (2016-2020) for Iran focused on reaching 90-90-90 goals by scaling up HIV testing and care services and developing more tailored programs for the key populations (7). During this time, Iran adopted the 2020 WHO guideline/test and treat for the initiation of antiretroviral therapy in HIV patients (8). This gives a pointer to increased resource (human, financial, institutional, material) and policy requirements for HIV management. Iran is an upper-middle income country with a GDP per capita of $5305 and health expenditure per capita of $ 1082 (9). This is a country having high financial commitments to managing its conditions of greatest burden, such as transportation accidents, natural disasters, non-communicable diseases, and many more diseases (10, 11).

As a result of the aforementioned problems, it is important to ascertain the affordability of the 2015/2020 ART initiation strategy for the national health systems of Iran. This was achieved using the generic disability-adjusted life-year (DALYS) framework provided by the global disease burden report. Previous studies have reported that the clinical and economic value of the 2020 guidelines was as a result of ART preventing transmission rather than treatment (12). As a result of this, results from a recent systematic review and meta-analysis regarding the risk of sexual transmission of HIV/AIDS with and without ART treatment were included as part of the input parameters.

Objective

The aim of this study was to determine the cost-effectiveness of alternative strategies for initiating antiretroviral therapy in HIV/AIDS patients in Iran.

Methodology

The Decision Analytic Model and Markov Model (3) were used to evaluate the clinical, economic consequences, and cost-effectiveness of the initiation of antiretroviral therapy in a model population of HIV/AIDS Iranian patients (Figure 1). The Markov model was based on a 5-year Markov state-transition cohort with annual cycle lengths and half-cycle corrections. The economic consequences were to the perspective of the national healthcare payers of Iran. The simulated populations were HIV positive individuals who all had a CD4 cell count >500 cells/ml. This population was categorized into two groups: Immediate arm: Defined by immediate initiation to ART. (Pathway A Figure1). This arm of initiation was made to simulate the 2020 WHO recommendations because it proposed the administration of ART to patients with CD4 cell count >500cells/ml.

Deferred therapy arm: Defined by administration of ART only when CD4 count falls below 350cells/ml or the patient develops AIDS symptoms. (Pathway B in Figure 1, B1 Received Therapy, B2 No therapy). This arm represents the old criteria for initiation which entails only those with CD4 cell count of below 350cells/ml to be administered therapy. Initiation to therapy is expected to occur at a rate of 14% per year and reach 70% by the fifth year (13,14).

Outcome measure

Our major outcome measure was disability-adjusted life years (DALY) averted, percentage DALY averted, costs at health payers'perspective, Incremental cost and Incremental cost-effectiveness ratio (ICER).

Disease model

Any patient who entered the model is expected to be followed for 5 years. Each arm of initiation was expected to be controlled or exhibit AIDS-related events such as tuberculosis, Kaposi sarcoma, malignant lymphoma or NON-AIDS-related events such as cancers, cardiovascular disease or unscheduled hospitalizations. These events were chosen to reflect the occurrence of both AIDS and NON-AIDS related event that are common to HIV patients across CD4 categories and geographical locations. They could also transmit infections or proceed to death (**Figure 1**). All events were assumed to be mutually exclusive.

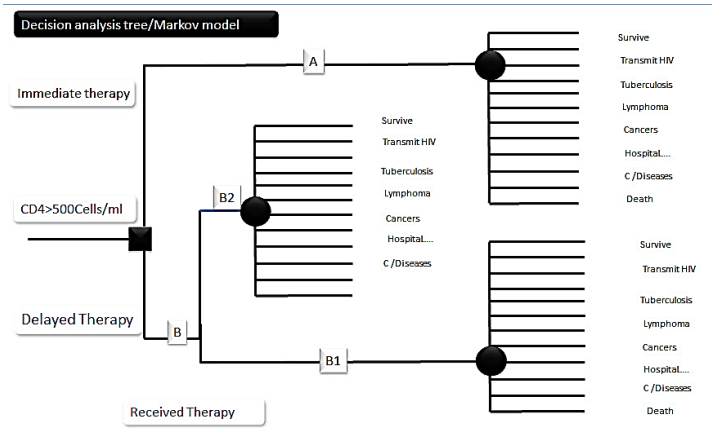


Figure 1. Decision Analytic Tree and Markov model

The patient in the deferred arm of initiation, in addition to all, could also get initiated to therapy if CD4 cell count reduces to <350cells/ml or they develop AIDS. Model structure and time horizon were carried out in reference to the START trial (14), the strategic timing of antiretroviral therapy. (A recent randomized controlled trial with same randomization of patients to ART therapy).

Model input data

Effectiveness measures

The input data (DALY) for computations were hazard rates from the START trial (14) and meta-analysis on the risk of sexual transmission of HIV. The disability weights were obtained from the global burden of disease study (15) (**Table 1**). (See Overleaf)

Table 1. Model Parameters for Disability Adjusted Life Year Computation.

Parameter		
Event	Hazard rates (14)	
	Immediate Therapy number/ 100 person years	Deferred Therapy number/100 person years
Mortality	0.17 (0.08-0.35)	0.30(0.15 -0.61)
Tuberculosis	0.09(0.03 -0.20)	0.28(0.12 -0.75)
Kaposi sarcoma	0.01(0.00 -0.11)	0.16(0.01 -1.00)
Malignant lymphoma	0.04(0.01 -0.15)	0.14 (0.00 -0.30)
Other cancers	0.13(0.06 -0.29)	0.26(0.12 -0.59)
Cardiovascular Disease	0.17(0.08 -0.36)	0.20(0.09 -0.43)
Other hospitalizations	3.94(3.33 -4.64)	4.31(3.65 -5.04)
New transmission a	0`.85(0.28 -2.99)	5.6 (3.26 -9.62)
Disability weights (14)		
HIV	0.053(0.034–0.079)	Each year for lifetime
Tuberculosis	0.399(0.267–0.547)	6months
Kaposi sarcoma	0.2740.(199–0.411)	3months
Malignant lymphoma	0.274(0.199–0.411)	3months
Other cancers	0.274(0.199–0.411)	3months
Cardiovascular Disease	0.082(0.053–0.117)	2months
Other hospitalizations	0.053(0.033–0.081)	2weeks
New transmission Treated	7.2	7.2, incurred one time at
Discount rates	0.03(0.00 -0.06)	time of infection

The hazard rates were converted to transition probabilities using the natural exponential function and subsequently converted to prevalence rate for each event in the two major arms. Prevalence rates were then computed to DALYs for each arm using standard methodology (**Figure 2**) (15). All events were assumed to be mutually exclusive.

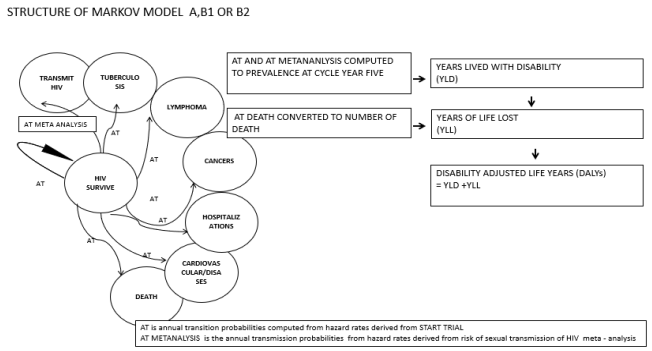


Figure 2. Markov Model and DALY Computation

Cost measures

The perspective of the national health payers of Iran was considered for collation of annual ART cost per person. The Iranian Research Center for HIV/AIDS at Imam Khomeini Hospital Tehran was the site of this data collection. The sample size of patients for both ART and PRE-ART stage were computed using the fisher's formula and 322 was derived for each group. Only direct medical costs were considered such as cost of medications, vaccines, diagnostic tests and personnel cost. Socio-demographic data of patients, diagnostic tests, medications, vaccines and their duration of therapy were obtained from the patient case notes using systematic random sampling at the center while the prices were obtained from the Iranian food and drug administration. The total annual ART cost per person was then computed after conversion from the Iranian rials to dollars based on the conversion rate of 114,500 IRR to one United State Dollar (USD).

The cost of ART per prevalent case at the end of year 5 represented the cost adduced to patients who received ART. This was in addition to the cost of other events. The cost of PRE-ART represented the cost adduced to patients who did not receive ART. The cost of death, new transmissions (discounted) and cost of each clinical event were also included as shown in **Table 2**. All these were obtained from relevant published literature (16), and were adjusted to the present value of 2020 USD. The GDP per capita was obtained from relevant published sources (9) and life expectancy was obtained by finding the life expectancy of a 20 years old HIV positive patient with CD4 greater than 200cells per ml in Iran (17,18). This life expectancy was chosen to align with 25 years which was the average age for the commencement of ART among HIV patients for Iran. The outcome measures were then computed based on standard methodology (15).

Table 2 Model Input Parameters for Cost Computation

S/N	Model Input Parameters	cost
1	ART	$470.00
2	Cost of pre-ART care	$129.00
3	Annual ART drug and management Cost	$599.25
4	Cost of tuberculosis	$1553.42 (26)
5	Cost of Kaposi's sarcoma	$1609.00 (26)
6	Cost of malignant lymphoma	$2030.00 (26)
7	Cost of other cancers	$1065.00 (26)
8	Cost of Cardiovascular Disease	$1065.00 (27)
9	Cost of other hospitalizations	$2451.00 (28)
10	Cost of death	$141.00 (29)
11	Cost of new HIV infection, (discounted)	$6847.00 (1)
12	Average local life expectancy, years	37.00 (9)
13	GDP per capita, in 2019 US$	$5305.00 (9)

Sensitivity Analysis

Deterministic one - way sensitivity analysis

A deterministic one - way sensitivity analysis was carried out by 10%, 20% then 50% increase followed by a decrease in the value of the base case incremental cost and then DALY averted to produce a range around the base case incremental cost-effectiveness ratio (ICER) which was reported as tables and tornado diagrams (**Figure 3**).

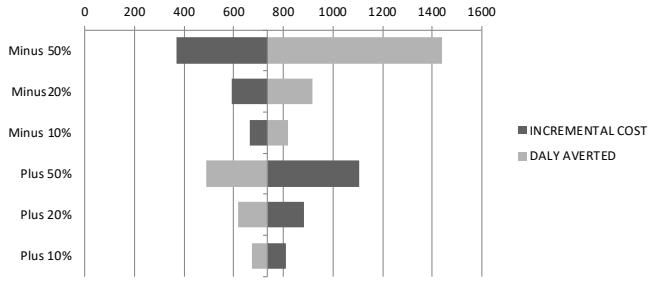


Figure 3. Tonado Analysis for Incremental Cost Effectiveness Ratio for Iran

Monte Carlo simulations

Monte Carlo simulations were conducted using 1000 iterations of the base case incremental cost and Daly averted. The probabilities of the ICERs being dominant and being cost-effective were found.

Statistical analysis

Statistical analysis was done using the Statistical Package for Social Scientist version 22.

Results

The prevalence of the disease was highest among the age group 15-49 years, according to socio-demographic data from an analysis of annual ART cost per person. Prevalence by sex was found to be 29% for women and 71% for men. The annual ART cost per person was $599.25 with a breakdown of $470 for ART cost and 129.25 for PRE- ART costs. The main cost driver for this consideration was found to be medications which composed of 74%, Diagnostic test and vaccines had 25% and personnel cost 1% of total costs. The Meta-analysis for the risk of transmission showed a decrease from 5.6PY (3.26-9.62) in untreated groups to 0.85 PY (0.28-2.99) in treated groups (**Figure 4a and 4b**).

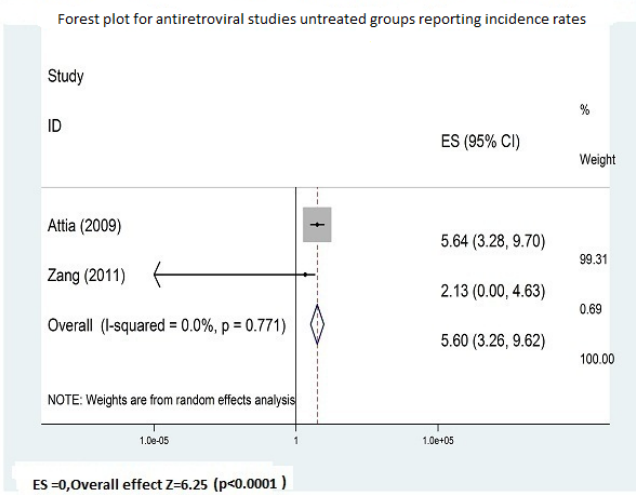
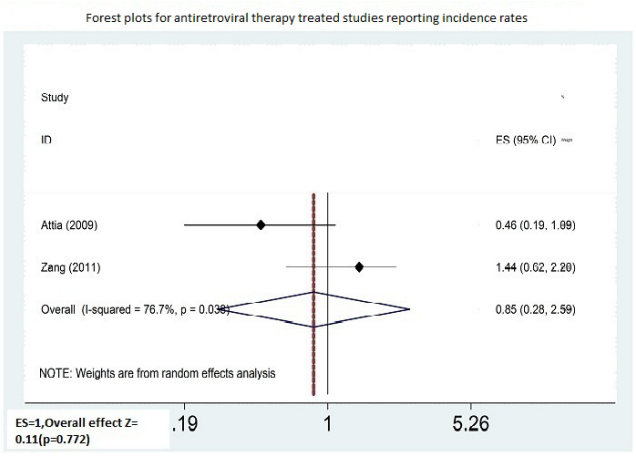


Figure 4a. Forest Plots for Anti-retroviral Therapy Untreated Groups



Figre 4b. Forest Plots for Antiretroviral Therapy Treated Groups.

The DALY averted (over 5 years) was found to be 0.31 due to the immediate arm exhibiting less burden of disease in terms of morbidity and mortality. This shows that the immediate arm had fewer clinical events, hence, less years of life lost and less years lived with disability (**Table 3**). At the end of 5 years, the incremental cost for management of the disease was found to be -$303.29 (**Table 3**). This was attributed to more clinical events for the deferred therapy arm, described by the higher prevalence of both AIDS and NON-AIDS-related events, translating to a higher financial burden for the old methods.

Table 3. Cost Effectiveness Analysis Base Case Results for Iran

	Immediate ART arm	Delayed ART arm	
Cost per patient($)	$1,352.71	$1,656	
Incremental cost ($)			$303.29
Effectiveness (DALYs)	-0.04	0.27	
DALY averted			0.31
Percentage DALY averted			115%
Incremental cost effectiveness ratio (Cost per Daly Averted $)			-$978.35
Probability of ICER dominant<0			
Probability of ICER<one GDP/ Capita			98.9%

The immediate therapy method of initiation accounts for a 115% reduction of the burden of disease. This shows the immense benefit of this recent innovation. The cost per DALY saved or incremental cost-effectiveness ratio of $978.35 (Negative ICER) is an indication of the dominance(cost-effectiveness) of the immediate therapy arm when compared with the deferred therapy arm.

The results of the deterministic one - way sensitivity analysis showed that all base case variations produced ICER that were still cost-effective (< GDP/capita of Iran$5305), putting the WHO threshold into consideration (**Table 4**) and as shown by the tornado diagram (**Figure 3**). The major driver of ICER variation was found to be a 50% reduction in DALY averted. The 1000 iterations in ICER had the probability of the ICER (dominant < 0) as 99% and probability of the ICER cost effective (< GDP/capita of Iran $5305) as 95.6% (**Table 3**).

Table 4. Cost Effectiveness Sensitivity Analysis Results for Iran

Immediate versus deferred therapy	Incremental cost	DALY averted (effectiveness)	Incremental cost effectiveness (ICER) Cost per Daly saved
-	-303.29	0.31	-978.3548387
Decrease in Incremental cost			
A[1]	273.051	0.31	880.8096774
B[2]	242.712	0.31	782.9419355
C[3]	151.695	0.31	489.3387097

Increase in Incremental cost			
A[1]	333.729	0.31	1076.545161
B[1]	364.068	0.31	1174.412903
C[1]	455.085	0.31	1468.016129
Decrease in Daly averted			
A[1]	-303.29	0.279	-1087.060932
A[2]	-303.29	0.248	-1222.943548
A[3]	-303.29	0.155	-1956.709677
Increase in Daly averted			
A[1]	-303.29	0.341	-889.4134897
B[2]	-303.29	0.372	-815.2956989
C[3]	-303.29	0.465	-652.2365591

Discussion

The clinical and economic consequences of initiation of antiretroviral therapy were evaluated using the recent 2020 guidelines for Iran. The first major finding was the meta-analysis that revealed an 84% reduction in risk of sexual transmission while on ART therapy, which may translate to a positive impact at the community level as evidenced (19). Although, the same study recommended cluster randomized controlled trials at the population level to verify their findings. An 84% reduction in the incidence of new infection would translate to reduction in spread of the disease (16) and as such, the 2020 WHO guidelines will serve as a preventive measure. This would also lead to overall cost savings to healthcare payers and increased productivity for individuals, with consequent additional income to families.

In addition to this, considering the cost of medicines, tests, and personnel needed to manage HIV/AIDS patients at the Iranian HIV/AIDS Research Center at Imam Khomeini Hospital Iran the cost of treating HIV/AIDS was estimated to be $599.25 per prevalent case. Comparable studies carried out in 2010–2011 for the same center estimated the per prevalent therapeutic cost to be $782 (20), and the cost requirements for a combination of research, training, and services to be $1494 per prevalent case per year. Differences in therapeutic costs could be explained by recent reduced foreign aid and reduced budgets (1) allocated for the management of these patients by the government of Iran.

The finding for this study that indicated that the gender more affected by the disease is male agrees with other studies for Iran as evidenced by (5). In this study it was stated that majority of this patients for Iran were male with less females having the disease. Most of the infected females are known to have acquired the disease from their male sexual partners who were people living with injection drug use (PWID) (5) .This is more so since the most common route of transmission for Iran is injection drug use (65.8%) and about 1,100 new HIV infections occur annually among sexual partners of PWID, putting them as the second-rank of HIV affected population in Iran" (5).

The above finding is contrary to the findings for Nigeria for which was discovered that the number of females having the disease was 58% of the total number of patients (3). This results from cultural practices that restricted most females' access to formal education and other forms of gender inequality. This leads to information asymmetry among the females regarding this disease as a result more infections occurred among females. This is bearing in mind the background that the major route of transmission for Nigeria is heterosexual intercourse. Furthermore, in the current study, the immediate therapy dominated the deferred therapy arm by being more effective and less costly. This was found to be consistent with recent studies (3,18, 21). All these studies implicated the new recommendations as having more clinical and financial benefits.

The new recommendations being cost effective does not translate to immediate overall cost saving to the Iranian healthcare payers as costs of disease management are bound to be on the rise over a couple of years due to efforts to increase access to ART, increase in life expectancies of the patients, the presence of co-morbid conditions as the patients live longer. This notwithstanding, there would be cost savings as a result of decreases in number of new infections and other disease related costs. There is the likelihood of this savings offsetting disease management cost in future.

The present study also revealed a negative ICER (-$978.35) which indicates dominance of the immediate over the deferred arm. This finding is significant because it implies Iran is handling the epidemic properly as the test and treat strategy was found to be cost-effective. This would go a long way to reduce the spread of the disease because this strategy is already being combined with other methods of combating the disease for Iran. Such interventions as establishment of Drop-in Centers Shelters, Outreach teams, Methadone treatment centers, Most-at-risk women's counseling centers, Counseling posts, Voluntary Counseling and Testing Centers (triangular clinics), Telephone hotlines and Positive Clubs (5) combined with the test and treat approach to therapy has been contributing to a reversal of the scourge in the recent years. These efforts would stimulate public confidence in Government programs/ interventions and uphold the credibility of health care delivery system.

This study has gone a long way to demonstrate that for the Iran health system, the 2020 WHO recommendations for initiation of antiretroviral therapy are cost-effective and would enhance the achievement of the Joint United Nations Program on HIV/AIDS (UNAIDS) 90-90-90 targets for ending the HIV epidemic which aimed to achieve the following; 90% of PLWH knowing their status, 90% of PLWH who know their status should be on treatment and 90% of PLWH on treatment should be virally suppressed by 2020.

It is worthy of note that UNAIDS 90-90-90 targets cannot be achieved by implementation of this guideline alone. At the population level, success can only be achieved when there is widespread testing uptake, linkage with care, uptake of therapy, retention of these in therapy, adherence to medications and a favorable social context for the occurrence of all these (22) (23). There is also need for reduction of the time between diagnosis and initiation of therapy (21).

Conclusion

The test and treat approach to initiation of antiretroviral therapy is cost-effective for Iran and when used in conjunction with other educative and preventive methods would go a long way to achieving reduction in HIV spread.

Limitations

We used a model that projected the costs and consequences over five years but could not project for more than 5 years. The model did not put into consideration that the transition probabilities for events could vary over the 5 years. In real-world situations, the asymptomatic patients with CD4 counts more than 500 cells/ml may not be motivated to give strict adherence to therapy. The direct medical cost which was achieved from Iranian research center for HIV/AIDS served as a proxy for Iran but this study did not put into consideration that some of this patients do not receive medications from the center.

Funding source

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Conflict of Interest

In the course of this research, there were no conflicts of interest.

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References are to be cited using Vancouver style. Citations must appear in order of appearance in the text with square brackets after the end of a sentence, i.e., [3]. The citation must electronically refer to the Reference Listing at the end of the manuscript.

References cited only in tables or in legends to figures should be numbered in accordance with a sequence established by the first identification in the text of the particular table or illustration. Figures must be labelled at the bottom, whilst tables shall be labelled at the top.

The number of references should normally be restricted to a maximum of 25 for a full paper, whereby not more than 20% should be not more than 5 years old, and no more than 10% should be more than 10 years old. References older than 10 years should ideally be classical subject material references.

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