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Efficacy of *aloe Secundiflora* crude extract against *Candida albicans* and *Penicillium marneffei*

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ABSTRACT

Medicinal plants have been used in production of various drugs singly or in combination and even as principal raw material for the production of other conventional medicines. There are a number of Aloe species that grow wildly in Kenya. Ten mature leaves of Aloe secundiflora were collected and the crude plant extract was obtained and prepared according to Waihenya et al., 2003. It was then tested for antifungal activity against Candida albicans and Penicillium marneffei. In- vitro studies on the efficacy of crude extracts of A. secundiflora on C.albicans and P. marneffei were conducted. The results of the study revealed considerable zones of inhibition of Candida albicans growth caused by Aloe secundiflora crude plant extract on Sabouraoud agar. There was also growth inhibition of the penicillium marneffei which is known to cause infections in immunosuppressed hosts and in non-AIDS patients with hematological malignancies and those receiving immunosuppressive therapy. Our results suggests that, the extract can be used to prevent the aflatoxins caused by Aspergillus species and the superficial mycosis that result from fungal infection and probably any other kind of mycoses. Further studies are required starting with experimental models to establish the in-vivo activity of the crude extract, the active ingredient, dosage and safety of A.secundiflora, before recommending it for clinical use.

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Introduction

Plants are a major source of medicinal compounds. Medicinal plants are used in many countries around the world and they play a pivotal role in provision of primary health care such as treatment of some communicable and non-communicable diseases. The use of herbal medicinal plants has always played a positive role in the control or prevention of diseases such as diabetes, heart disorders and various cancers (Mohanta. B., et al 2003). Advanced microbial and chemical methods can synthesize medicinal and aromatic compounds, but usually the costs are high. Conventional medicine is known to cause side effects, as a result, some people end up preferring various herbal mixtures. Some of the herbal mixtures used are quite effective because they contain specific substances that have proven physiological activity. There are over 500 different varieties of Aloe species commonly used including A. perryi from Socotra Island or Zanzibar and A. ferox from Africa. There are a number of Aloe species that grow wildly in Kenya. Herbalists around Lake Victoria region in Kenya have used Aloe Secundiflora in treating ailments including; chest problems, polio, malaria and stomach ache (Kigondu EVM et al., 2009).

Aloe products are available in form of creams, tablets, capsules, powders, gels as well as distilled liquid or "juice". It is extracted from aloe plant, and reconstituted. Aloe barbadensis (Aloe vera) is the most commonly used species. Aloe is native to East and South Africa and is grown in most subtropical and tropical locations, including Latin America and the Caribbean. The major species is Aloe vera (Aloe barbadensis Miller). Aloe are leaved perennial herbs, shrubs or trees, with leaves double ranked or in rosette, usually more or less triangular or sickle shaped, the margins usually armed with sharp teeth, normally with bitter tasting yellow, brown liquid when broken. They are popular garden and pot plants (Aguilar and Brink, 1999).

Two products were obtained from the Aloe leaves, both of which have been medicinally used for centuries. The fraction called gel of parenchyma cells, which is colourless and tasteless has been used particularly for treatment of skin diseases (Grindaly and Reynolds, 1986). In addition to a large amount of water, the gel mainly contains polysaccharide (Femenia et.. al., 1999), of which the acetylated mannose sugar is the major bioactive component, commercially known as ACEMANAN or CarasynTM (Mc Daniel et.al., 1987).Other components of the gel include aluminium, boron, calcium iron, Manganese, sodium phosphorous, silicon and strontium. The exudates fraction consists of phenolic compounds mainly chromones and anthrones (Park et. al., 1998; Kuzuya et. al., 2001) of which an anthrone C-glucoside, barbaloin (aloin A), is the major component (Zonta et. al.,1995). Aloe secundiflora is used for both ethnomedical and ethonoveterinary purposes. Some of the local uses include anti-venom, anti-parasitic and anti-diabetic as well as for making alcoholic preparations bitter (Waihenya et. al., 2003). The leaf exudates of this species have found ethnoveterinary use for management of some viral disease. (Ethnoveterinary medicine in Kenya, 1996). In poultry for example, the exudates has been extensively used as prophylaxis for Newcastle disease (ND) virus and therapeutic for fowl typhoid, coccidiosis and other enteric conditions (Waihenya, 2002, Waihenya et. al., 2003). Aloe secundiflora has also been used traditionally for the treatment of salmonellosis, lumpy skin disease wounds and eye infections in cattle, sheep and goats (Waihenya, 2002).

The organisms of the fungal lineage include mushrooms, rusts, musts, puffballs, truffles, morels, moulds and yeast as well as many less well-known organisms. There are many species of the genus Candida that cause disease. The infections caused by all species of Candida are called candidiasis. Candida albicans is an endogenous organism. It can be found in 40 to 80% of normal human beings. It is present in the mouth, gut and vagina. It may be present as a commensal or a pathogenic organism. Infections with Candida usually occur when a patient has some alteration in cellular immunity, normal flora or normal physiology. Patients with decreased cellular immunity have decreased resistance to fungal infections. Prolonged antibiotic or steroid therapy destroys the balance of normal flora in the intestine allowing the endogenous Candida to overcome the host. Invasive procedures, such as cardiac surgery and indwelling catheters, produce alterations in host physiology and some of these patients develop Candida infections. The increasing magnitude of antifungal resistance as well as the advent of new antifungal drugs has generated a renewed interest in fungal susceptibility testing. Candida albicans is the best studied of the human fungal pathogens, and it serves as a model organism for the study of other pathogenic fungi. In recent years, the frequency of fungal infections has steadily grown and although these infections are generally less frequent than bacterial infections, at least two aspects make them increasingly important. First, opportunistic infections in immunocompromised patients represent an increasingly common cause of mortality and morbidity. Second, many of the currently used antifungal compounds are often of limited use because of their toxicity and side effects (Edwards, J. E., 1991). Furthermore, within the last decade there has been an emergence of anti-fungal drug resistance, which was a rarity in the past (Chander.J, 2002).

The common occurrence of Penicillium species in food is a particular problem. Some species produce toxins and may render food inedible or even dangerous. On the other hand, some species of Penicillium are beneficial to humans. Cheese such as roquefort, brie, camembert, stilton, etc. are ripened with species of Penicillium and are quite safe to eat. The drug penicillin is produced by Penicillium chrysogenum, a commonly occurring mould in most homes. Penicillium spp. are occasional

causes of infection in humans and the resulting disease is known generically as penicilliosis. Penicilliosis is an infection caused by Penicillium marneffei, a dimorphic fungus endemic to Southeast Asia and the southern part of China. Persons affected by penicilliosis usually have AIDS with low CD4+ cell count of typically <100 cells/cu mm. The average CD4 count at presentation is 63.5 cells/cu mm. Penicillium marneffei infections have also been reported in non-AIDS patients with hematological malignancies and those receiving immunosuppressive therapy. Penicillium marneffei infection, so called penicilliosis marneffei, is acquired via inhalation and results in initial pulmonary infection, followed by fungemia and dissemination of the infection. The lymphatic system, liver, spleen and bones are usually involved. Acne-like skin papules on face, trunk, and extremities are observed during the course of the disease. Penicillium marneffei infection is often fatal. Patients with penicilliosis are occasionally seen outside endemic areas, but most have a history of travel to an endemic area. The most common presentation is a disseminated infection manifested by fever, skin lesions, anemia, generalized lymphadenopathy, and hepatomegaly. Localized infection such as pneumonia has also been reported. Penicillium has been isolated from patients with keratitis, endophtalmitis, otomycosis, necrotizing esophagitis, pneumonia, endocarditis, peritonitis, and urinary tract infections. Most Penicillium infections are encountered in immunosuppressed hosts. Corneal infections are usually post-traumatic.

Materials and Methods

Plant collection, sample extraction and preparation

Aloe secundiflora was obtained from various areas to ensure proper sampling. The leaves were collected by plucking them from the points attached to the stem taking care not to damage them. This was done when the leaves were still fresh. Ten leaves were then plucked from different plants at their base, transverse sections were made and they were vertically arranged in a container with distilled water. After an hour, the exudates were sieved and lyophilised at 0.1 bar at 700C for 48 hrs to obtain a dry yellow/orange powder (Waihenya et. al., 2003). Serial dilutions were carried out from the powder obtained at different concentrations starting from a stock solution of 2000mg/ml of the crude up to 100mg/ml.

Test microorganisms

Candida albicans ATCC 10231 and Penicillium marneffei were maintained on Sabouraud agar slants and transferred at regular intervals. The fungal species were cultivated and sub cultured on Sabouraud agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India) by overnight incubation at 370C, which was sufficient to prevent formation of mould form of Penicillium marneffei. A repeat subculture on Sabouraud agar was made to ensure purity and viability. The pure colonies of the test organisms were then grown in a sterile environment whereby they were then suspended in sterile normal saline and vortexed. The organisms were later subjected to the prepared Aloe sensitivity discs as a way of screening for antifungal activity.

Preparation of Aloe Secundiflora sensitivity discs

Discs of six milliliters were prepared from Whatman no.1 filter paper. The discs were then sterilized by autoclaving. After sterilization, the discs were dried on hot air oven at 50°C for two hours to remove the excess moisture. Different Aloe concentrations were prepared to which the discs were then soaked with about 20 ml of each sterile Aloe concentrations. They were put in sterile petri dishes grouped according to their concentrations and left to dry completely at 37oC for 24 hours. The inoculum used for screening studies was prepared by adjusting the concentration of microorganisms in sterile water for injection using spectronic-20 colorimeter (Bausch and Lomb) set at 630 nm with 65% transmittance. Sets of petridishes were washed, rinsed with sterile distilled water, dried, wrapped in aluminium foil and sterilized using an autoclave. Paper discs that were impregnated with methanol and air-dried were used as negative controls. Fluconazole discs (10 µg) obtained from Mast Laboratories (Bootle, UK) were used as the positive control antifungal agent.

Sensitivity testing

Two methods were used to assess the sensitivity of C. albicans and P. marneffei to various concentrations of the crude extract.

(a)Paper disc method

The inoculum was standardized by preparing it to an equivalent of 0.5 McFarland standard. Using sterile cotton swabs, the Sabouraud dextrose agar (bioMérieux) was inoculated uniformly by the streaking method with the prepared sets of inoculum under aseptic conditions. Sabouraud dextrose agar, was chosen because it is the routine medium for primary isolation. Using sterile forceps, the paper discs containing different concentrations of Aloe secundiflora crude plant extract were applied at equal distances from each other on plates that had been inoculated with C. albicans and P. marneffei. The inverted plates were inoculated for 24 hrs at 370C. Application of different discs that had been impregnated with varying concentrations of the crude extract were then carried out followed by application of fluconazole, which in this case was the positive control. Each impregnated disc was carefully placed on to the inoculum to ensure complete contact with the agar surface.

(b) Well method

In prepared potato dextrose agar, wells were punched using a sterile teat pipette. They were labelled at the bottom of the petri dishes according to the concentrations of the crude extract prepared. Using sterile cotton wool swabs, culture solutions of the test micro organisms were evenly spread on the media surface. The appropriate dilutions of Aloe crude extract were dispensed into the wells using sterile needles and syringes taking care of the spill over of the wells. 50 ml of the crude extract was dispensed into each well and the plates were incubated at 37°C for 24 hours.

Measurement of inhibition zones

Following incubation, the diameter of the zones of inhibition (excluding the size of the well and paper disk) were measured in millimetres (mm).

Results

(a) Paper disc method

Average measurements (mm) of the diameter of the zones of inhibition from different concentrations of the Aloe crude extract using the paper disc sensitivity method after carrying out the experiments in five triplicates are shown in Table 1.

Micro organism	*Aloe secundiflora* concentration (mg/ml)						Positive control (25µg)
	100	250	500	1000	1500	2000	
Candida albicans	0.5	1	2	3	3	5	18.0
Penicillium marneffei	0.0	0.5	1	1.5	2	2.5	6

Table 1: *Inhibition diameters (mm) of C.albicans and P.marneffei against different concentrations of A.secundiflora using the paper disc method.*

The positive control antifungal agent used was Fluconazole (10µg).

(b) Well method

Micro organism	*Aloe secundiflora* concentration (mg/ml)						Positive control (25µg)
	100	250	500	1000	1500	2000	
Candida albicans	0.5	2.0	3.5	6.0	5.0	7.0	18.0
Penicillium marneffei	0.0	1.0	1.5	2.0	3.0	4.0	5.0

The average measurements (mm) of the zones of inhibition using the punched well method after carrying out the experiments in five triplicates are summarised in Table 2.

Table 2: *Inhibition diameters (mm) of C.albicans and P.marneffei against different concentrations of A.secundiflora using the well method*

The positive control antifungal agent used was Fluconazole (10µg). The recorded zones of inhibition excluded the diameter of the wells in which the preparations of the Aloe secundiflora crude extract was placed.

Discussion

Candida albicans was the most susceptible of the two organisms. With respect to the growth rate Candida grew faster than Penicillium marneffei. Candida appeared to have mucoid, white, single and round colonies. Penicillium marneffei initially formed white colonies that later grew into a fine uniform green mass on the agar. Antifungal effect was shown by considerable zones of inhibition around the wells and the paper discs (Figure 2). Fluconazole, which was used as the positive control and for comparison, was more effective in terms of activity since its inhibition zones were much bigger, compared to crude extract's inhibition. Despite the antifungal activity that was displayed in this study, it was evident that A.secundiflora crude extract was required in greater concentrations for the extract to achieve similar effects in terms of growth inhibition as the positive control that was used. There was a significant variation in the zones of inhibition at the different concentrations. In addition, there was an increase in the zone of inhibition as the concentration of the crude extract increased. The highest zone of inhibition of growth for C.albicans using the well method was 7.0mm ± 1.09. This was obtained under the highest concentration of the extract which was 2000mg/ml. In comparison, the highest zone of inhibition of growth for the same fungi using the paper disc method was 5.0mm ± 1.09 at the same concentration of 2000mg/ml.

Candida is a common cause of fungal infections in man and other domesticated animals. In the past a lot of people have used the crude extract of Aloe to treat skin ailments most of which are fungal infections. Serious fungal infection carries considerable morbidity and mortality. Patients infected with HIV contribute to the majority of cases associated with this increase and the use of long-term antifungal therapy in these patients and others gives rise to an increase in the frequency of resistance to antifungals. This has highlighted the need for easy, rapid and reliable antifungal susceptibility tests (Wey et al., 1988). Previous studies (Waihenya, 2002) have shown that the crude extract contains antibacterial effect including that of the bacteria that cause skin infections such as Staphylococcus aureus. The study showed that crude extract of Aloe Secundiflora has an antifungal activity on fungi such as C. albicans and P. marneffei. However, further research should be done on the anti fungal activity of A. secundiflora against many other species of fungi.

Conclusions

Further studies to determine the active biomolecules in Aloe secundiflora and their mode of action are necessary. Toxicological assessment of the crude extract

should be established especially since high concentrations of the extract attain substantial antifungal activity. Finally, this study shall corroborate the use of Aloe secundiflora crude plant extract for management of fungal infections

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