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Full Length Research Paper

Phytochemical extraction and antimicrobial properties of different medicinal plants*: Ocimum sanctum* (Tulsi), *Eugenia caryophyllata* (Clove), *Achyranthes bidentata* (Datiwan) and *Azadirachta indica* (Neem)

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Aqueous ethanolic extract of four medicinal plants were subjected to *in vitro* antibacterial assay against human pathogenic *Escherichia coli, Salmonella typhi, Salmonella paratyphi, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* employing cup diffusion method. Among four plants tested *Eugenia caryophyllata* (Clove) was found to be the most effective against *S. typhi*. All the plants were ineffective against *E. coli* and *K. pneumonia. Achyranthes bidentata* was found to be ineffective against all the tested organisms. The largest zone of inhibition (22 mm) was obtained with *E. caryophyllata* against *S. typhi* and Minimum Bactericidal Concentration (MBC) value of 5 mg/l was obtained with *Azadirachta indica* against *S. typhi. K. pneumoniae* and *E. coli* were found to be resistant with all the plant extracts. A qualitative phytochemical analysis was performed for the detection of alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins and reducing sugars. Thin layer chromatography was also performed using solvent system chloroform, methanol and water (10:10:3) for the analysis of lipid present in plant extract. The present study will be successful in identifying candidate plant with different antimicrobial activity which could be further exploited for isolation and characterization of the novel phytochemicals in the treatment of infectious disease especially in light of the emergence of drug-resistant microorganisms and the need to produce more effective antimicrobial agents.

Key words: Antibacterial property, drug resistance, medicinal plant, zone of inhibition.

INTRODUCTION

Plants are the richest resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Hammer et al., 1999). The use of plants and plant products as medicines could be traced as far back as the beginning of human civilization. The earliest mention of medicinal use of plants in Hindu culture is founds in “Rigveda”, which is said to have been written between 4500 - 1600 B.C. and is supposed to be the oldest repository of human knowledge. It is Ayurveda, the foundation of medicinal science of Hindu culture, in its eight division deals with specific properties of drugs and various aspects of science of life and the art of healing (Rastogi and Mehrotra, 2002).

Medicinal plants are a source of great economic value all over the world. Nature has bestowed on us a very rich botanical wealth and a large number of diverse types of plants grow in different parts of the country. Nepal is rich in all the 3 levels of biodiversity, namely species diversity, genetic diversity and habitat diversity. In Nepal thousands of species are known to have medicinal value and the use of different parts of several medicinal plants to cure specific ailments has been in vogue since ancient times. Herbal medicine is still the mainstay of about 75 - 80% of the whole population, and the major part of traditional

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therapy involves the use of plant extract and their active constituents (Akerele, 1993). Among the 7,000 species of medicinal plants recognized all over the world, more than 900 types of precious medicinal plants are said to be found in Nepal (Manandhar, 2000). Unfortunately, only few of them are used for their medicinal value. Our approach involved to explore the antibacterial activity of four medicinal plants and study their antimicrobial constituents.

About 1500 plants are systematically used in indigenous system of medicine, like Ayurveda, Unani and Siddha. However, the ethnopharmacologists, botanists, microbiologists and natural-product chemists world over today, is constantly still in search of medicinal efficacy of plants and their phytochemicals, since the reported data so far available on plants are comparatively meager before the vast number of plant population. The drugs which are already in use to treat infectious diseases is of concern because, drug safety remains an enormous global issue. It was estimated that 2.22 million hospitalized patients had serious Adverse Drug Reactions (ADR) and 106,000 died in a single year in the USA. This Herbal and natural products have been used in folk medicine for centuries throughout the world, but there are relatively lower incidences of adverse reactions to plant preparations compared to modern conventional pharmaceuticals, this coupled with their reduced cost, is encouraging for both the consuming public and national health care institutions to consider plant medicines as alternatives to synthetic drugs (Nair et al., 2005).

Nepal is rich in varieties of medicinal plants. Among the 7,000 species of medicinal plants recognized all over the world, more than 900 types of precious medicinal plants are said to be found in Nepal (Manandhar, 2000). Unfortunately, only few of them are used for their medicinal. The plant parts that are undertaken under study viz Neem, Tulsi, Clove and Datiwan, are mainly used by the indigenous Nepalese people for the various ailments mainly for the dental caries periodontal disease, asthma, inflammation and indigestion. Due to the lack of doctors, medication and expenses associated with the medicine local community of remote areas of Nepal like Jumla, Humla, Dadeldhura and Doti make their plant preparation for treating above mention disorders. So, our approach involved to explore the antibacterial activity of four medicinal plants and study their antimicrobial constituents.

MATERIALS AND METHODS

Collection of samples

The medicinal plants used for the experiment were leaves of *Ocimum sanctum* (Tulsi), flower of *Eugenia caryophyllata* (Clove), stem, leaves of *Achyranthes bidentata* (Datiwan), stem and bark of *Azadirachta indica* (Neem). The plant parts were identified according to various literatures, Medicinal Plants of Nepal by HMG/N (1976) including other pertinent taxonomic literature.

Collection of test organism and preparation of stock culture

Test organisms were received from Central Department of biotechnology, TU and Manmohan Memorial Hospital, Kathmandu and reconfirmed by Gram staining and sub culturing in appropriate selective media.

Solvent extract

Five gram of the plant powder was loaded in the thimble of Soxhlet apparatus. It was fitted with appropriate size round bottom flask with 250 ml absolute ethanol, and upper part was fitted with condenser. Constant heat was provided by Mantox heater for recycling of the solvent. After complete extraction, the extract in round bottom flask was transferred into clean and pre-weighed universal tubes. Universal tubes containing extracts were weighted and noted down and finally, the percentage yield was calculated. Percentage yield was calculated as dividing initial weight of raw material taken by final weight of extract.

Preparation of standard culture inoculum of test organism

Three or four isolated colonies were inoculated in the 2 ml nutrient broth and incubated till the growth in the broth was equivalent with Mac-Farland standard (0.5%) as recommended by WHO.

Separation of active compound from tulsi extracts suspension by preparative thin layer chromatography (TLC)

Preparation of chromaplate

The glass plates were cleaned and dried in hot air oven. Slurry was prepared by mixing silica gel (silica gel for thin layer chromatography incorporating 13% calcium sulfate as binder) by adding double the volume of distilled water in clean beaker with continuous stirring. One larger drop of slurry was placed on the slide and by using another clean slide edge the drop of slurry was scattered all over the slide to make thin film and left as such for sometimes. This procedure is applied for the preparation of all chromaplates in microscopic slide. The chromoplates were activated by heating them in the hot air oven at 120°C for 30 min.

Loading of sample

The plate was allowed to cool at room temperature and marked about 2 cm from the bottom as the origin. The working suspension was loaded at the center of the slide about 2 cm above from the edge.

Development of chromatogram

The development tank was saturated with suitable solvent system chloroform, methanol and water (10:10:3) for the analysis of lipid present in plant extract. The plate was kept in the tank without touching baseline by solvent and left for development. The final solvent front was marked and the plate was dried.

Spot visualization

Few pieces of iodine crystals were kept in the tank and covered with glass plate to saturate the tank with iodine vapor. The plate was then kept in iodine vapor saturated tank and left for few hours.

Collection of the active compound

Spots on the preparative silica gel plate were scratched with the help of clean and dry spatula and collected in beaker containing 70% ethanol and left overnight. The content in the beaker was stirred and filtrated through Whatman no. 1 filter paper. The filtrate was collected in clean and dry beaker. The filtrate containing active compound was used for the determination of antimicrobial effect.

Antibacterial activity assay

Antibacterial activity of the different extracts was determined by cup diffusion method on nutrient agar medium (Anon, 1996). Wells are made in nutrient agar plate using cork borer (5 mm diameter) and inoculums containing 10^6 CFU/ml of bacteria were spread on the solid plates with a sterile swab moistened with the bacterial suspension and fifty micro-liters of the working suspension/solution of different medicinal plant extract and same volume of extraction solvent for control was filled in the wells with the help of micropipette. Plates were left for some time till the extract diffuse in the medium with the lid closed and incubated at 37°C for 24 h. After overnight incubation the plates were observed for the zone of inhibition (ZI) and the diameter of the inhibition zone were measured using scale and mean were recorded.

Determination of minimum bactericidal concentration (MBC)

Freshly prepared nutrient broth was used as diluents. Crude extract was diluted by two fold serial dilution method. 50 µl of the standard culture inoculums was added to each test tube except the negative control tube. All tubes were incubated at 37°C for 24 h. The tube content was subculture in fresh nutrient agar separately and MBC was determined as that showing no growth.

Identification tests for active compounds

The tests were done to find the presence of the active chemical constituents such as alkaloids, glycosides, terpenoids and steroids, flavonoids, reducing sugar and tannin by the following procedure.

Alkaloid

Alkaloids are basic nitrogenous compounds with definite physiological and pharmacological activity. Alkaloid solution produces white yellowish precipitate when a few drops of Mayer's reagents are added (Siddiqui and Ali, 1997). Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer's reagent (Evans, 2002).The alcoholic extract was evaporated to dryness and the residue was heated on a boiling water bath with 2% hydrochloric acid. After cooling, the mixture was filtered and treated with a few drops of Mayer's reagent. The samples were then observed for the presence of turbidity or yellow precipitation.

Glycoside

Glycosides are compounds which upon hydrolysis give rise to one or more sugars (glycones) and a compound which is not a sugar (aglycone or genine). To the solution of the extract in glacial acetic acid, few drops of ferric chloride and concentrated sulphuric acid are added, and observed for a reddish brown coloration at the junction of two layers and the bluish green color in the upper layer (Siddiqui and Ali, 1997).

Terpenoid and steroid

Four milligrams of extract was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. Then concentrated solution of sulphuric acid was added slowly and red violet color was observed for terpenoid and green bluish color for steroids (Siddiqui and Ali, 1997).

Flavonoid

Four milliliters of extract solution was treated with 1.5 ml of 50% methanol solution. The solution was warmed and metal magnesium was added. To this solution, 5 - 6 drops of concentrated hydrochloric acid was added and red color was observed for flavonoids and Orange color for flavones (Siddiqui and Ali, 1997).

Tannins

To 0.5 ml of extract solution 1 ml of water and 1 - 2 drops of ferric chloride solution was added. Blue color was observed for gallic tannins and green black for catecholic tannins (Iyengar, 1995).

Reducing sugar

To 0.5 ml of extract solution, 1 ml of water and 5 - 8 drops of Fehling's solution was added at hot and observed for brick red precipitate. All the plant extracts were subjected to individual microbiological tests to ascertain their antimicrobial activity against three species of microorganism: *S. aureus*, *E. coli* and *K. species*. The antimicrobial activity of the extracts was determined by measuring the diameter of ZI exhibited by the extracts.

RESULTS

The Gram positive bacteria chosen for the study was *S. aureus* and Gram negative bacteria were *K. pneumoniae*, *E. coli*, *P. aeruginosa, S. typhi, and S. paratyphi.* Among four pants tested *E. caryophyllata* was found to be the most effective against *S. typhi*. All the plants were ineffective against *E. coli*. The largest zone of inhibition (22 mm) was obtained with *E. caryophyllata* against S*. typhi* and MBC value of 5 mg/l was obtained with *A. indica* against *S. typhi* (Table 1 and Figure 1). A qualitative phytochemical analysis was performed for the detection of alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins and reducing sugars. The percentage yield of *O. sanctum* was found to be 29.08%, *E. caryophyllata* (19.58%), *A. bidenta* (21.07%) *and A. indica* (17.15%) (Table 2 and Figure 2). Thin layer chro-matography was also performed using solvent system chloroform, methanol and water(10:10:3) for the analysis of lipid present in plant extract *A. bidentata* was found to be ineffective against all the tested organisms. *K. pneumoniae* and *E. coli* were found to be resistant with all the plant extracts.

DISCUSSION

The beneficial medicinal effects of plant materials

Table 1. List of plants and their parts used in the experiment.

S/N	Scientific name	Common name	Plant part used	Family	Phytochemicals	Properties
01.	*O.sanctum*	Tulsi	Leaves	Lamiaceae	Ursolic acid flavonoids such as apigenin, polyphenols, anthocyanins and luteolin, eugenol, thymol or sesquiterpene alcohols	Anti-inflammatory, antiarthritic, anti-stress and antipyretic(Singh et al.,1996)
02.	*E. caryophyllata*	Clove	Flower	Myrtaceae	Eugenol, acetyleugenol, chavicol, acetyl salicylate and humulenes (Zheng,et al., 1992)	Treat colds, dental abscesses, gum disease, earache and arthritis pain. anti-fungal, anticonvulsant (Harborne and Baxter, 1993), anticarcinogenic and antimutagenic activities (Miyazawa and Hisama, 2001)
03.	*A. bidentata*	Datiwan	Stem and leaves	Amaranthaceae	The roots contain triterpenoids, sitosterol and sigma sterol (Nguyen et al., 1989).	The root juice is used in Nepal in treatment of toothache. This juice is also used in the treatment of indigestion and is considered to be a good treatment for asthma (Manandhar, 2002). The stem of the plant is used as a toothbrush that is said to be good for the health and is also a treatment for pyorrhea (Manandhar, 2002). The leaves and stems are harvested in summer and are usually crushed for their juice or used in tinctures (Bown, 1995).
04.	*A. indica*	Neem	Stem bark	Meliaceae	Phenols, unsaturated sterols, triterpenes and saponine (Subramanian and Lakshmanan, 1996). Phenolic diterpenoids, limonoids), c-secomeliacins, c-secolimonoids, polysachharides etc. have also been isolated from bark (Fujiwara et al., 1984).	Leaf extract of neem shows antiviral activity against group B coxsackieviruses (Badam and Bedekar, 1993).The leaves are carminative and expectorant, anti-inflammatory, anti-rheumatic, useful in syphilitic sores, earache, boils, in all blood impurities. A decoction of neem leaves relieves nose troubles; heal wounds, used as a gargle in stomatitis and bad gums.

typically result from the secondary products present in the plant although, it is usually not attributed to a single compound but a combination of the metabolites. The medicinal actions of plants are unique to a particular plant species or group, consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct (Parekh et al., 2005). The screening of plants usually involves several approach; ethno botanical approach is one of the common methods that are employed in choosing the plant for pharmacological study.

Plant essential oils and extracts have been used for many thousands of years, in food preservation, pharmaceuticals, alternative medicine and natural therapies. It is necessary to investigate those plants scientifically which have been used in traditional medicine to improve

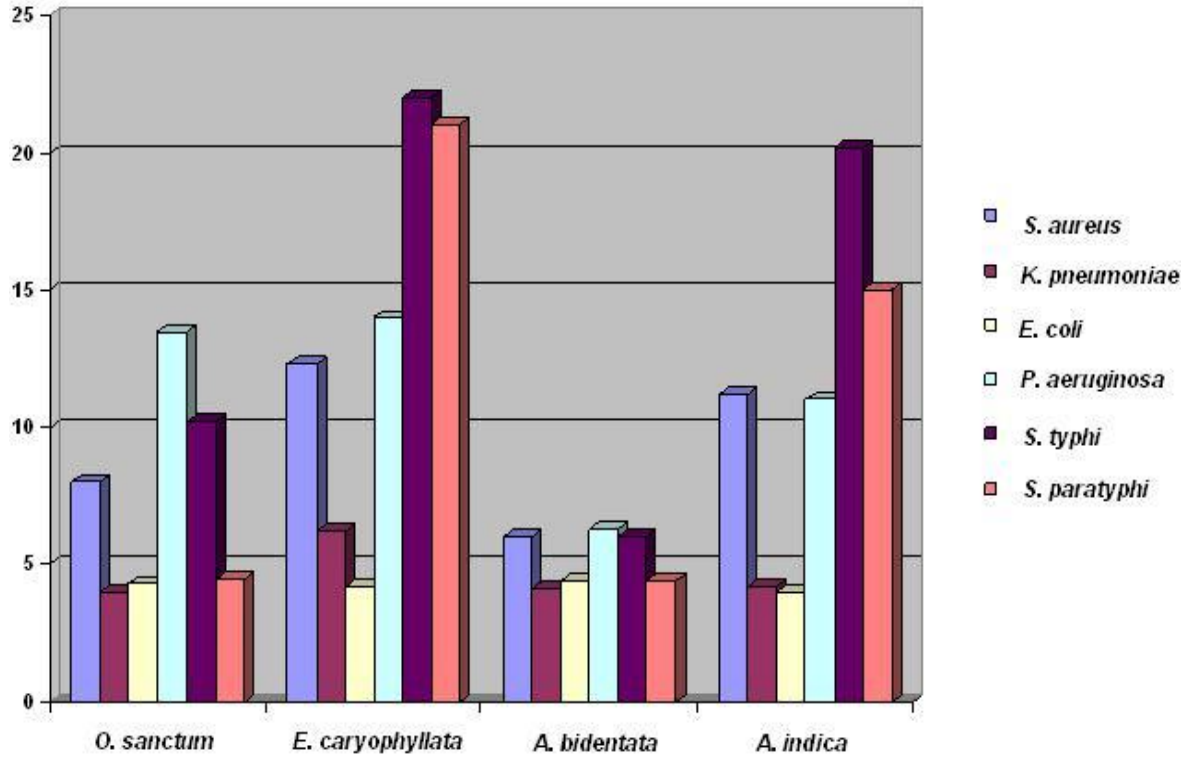

Figure 1. Graph showing zone of inhibition of different microorganisms against different plant extract.

Table 2. Phytochemical constituents of the plant extracts.

Plant extracts	Alkal-oids	Glyco-sides	Terpenoids and steroids	Flavinoids	Tannins	Reducing sugar
O. sanctum (Tulsi)	+ve	+ve	+ve (steroid)	-ve	+ve	-ve
E. caryophyllata (Clove)	-ve	+ve	-ve	-ve	+ve	+ve
A. bidentata (Datiwan)	+ve	+ve	+ve	-ve	-ve	+ve
A. indica (Neem)	+ve	+ve	+ve	-ve	+ve	-ve

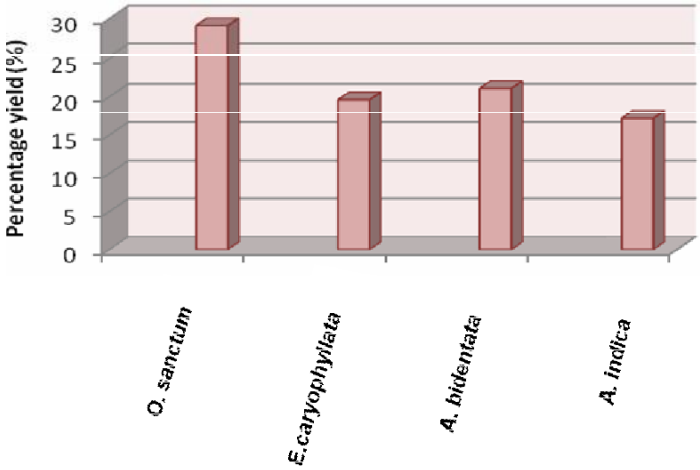

Figure 2. Graph showing percentage yield of different plant extract.

the quality of healthcare. Plant extracts are potential sources of novel antimicrobial compounds especially against bacterial pathogens. *In vitro* studies in this work showed that the plant extracts inhibited bacterial growth but their effectiveness varied. The antimicrobial activity of many plant extracts has been previously reviewed and classified as strong, medium or weak (Zaika, 1998).

The medicinal plants like Tulsi, Neem, Datiwan and Clove are being used traditionally for the treatment of inflammation, wound healing, carminative, cough, toothache, antiseptics expectorant, stomatitis and some fungal infection like candidaisis. The antibacterial activity has been attributed to the presence of some active constituents in the extracts. The phytochemical analysis of *A. indica* extract had earlier been reported (Kraus et al., 1981). Phytochemical screening of the stem bark extract of *A. indica* in the present study also revealed

Table 3. Observation of antimicrobial property of alcoholic extracts of different medicinal plants against different pathogenic microorganisms.

S/N	Test organism	Name of the plants			
		***O. sanctum* (Tulsi)**	***E. caryophyllata (*Clove)**	***A. bidentata* (Datiwan)**	***A. indica* (Neem)**
1	*S. aureus*	+	+	-	+
2	*K. pneumoniae*	-	-	-	-
3	*E. coli*	-	-	-	-
4	*P. aeruginosa*	+	+	-	+
5	*S. typhi*	+	+	-	+
6	*S. paratyphi*	-	+	-	+

presence of terpenes and glycosides. Study suggested a number of active constituents might be present in the neem bark extract to control gastroduodenal ulcers. However, a glycoside appeared to be the major bioactive component that offers antisecretory and antiulcer effects (Bandyopadhyay et al., 1998, 2002). Phytochemical screening of the stem bark extract of *A. indica* in the present study also revealed presence of terpenes and glycosides. Plant glycosides, which are not normally toxic when ingested orally, are known to inhibit chloride transport in the stomach (Machen and Forte, 1979). The neem oil, also known as oil of Margosa, is believed to have medicinal properties, such as antibacterial (Singh and Sastri, 1981) antifungal (Kher and Chaurasia, 1977) and antidiabetic (kraus et al., 1983).

An important characteristic of plant extracts and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable (Sikkema et al., 1994). The inhibition produced by the plant extracts against particular organism depends upon various extrinsic and intrinsic parameters. Due to variable diffusability in agar medium, the antibacterial property may not be demonstrated as ZOI commensurate to its efficacy. Therefore MBC value has also been computed in this study. MBC is the lowest concentration of antibacterial substance required to produce a sterile culture (Cheesbrough, 1993).

In classifying the antibacterial activity as Gram-positive or Gram-negative, it would generally be expected that a much greater number would be active against Gram-positive than Gram-negative bacteria (McCutcheon et al., 1992), however, in our study both Gram positive and Gram negative bacteria are found to be active against plant extract. According to the antibacterial assay done for screening purpose, among Gram negative bacteria *E. coli* and *K. pneuminae* are found to be resistant against all tested plant extract (Table 3). All of the plant extract except *A. bidentata* were found to be the most effective against gram positive bacteria *S. aureus,* a pyrogenic bacterium known to play a significant role in invasive skin diseases including superficial and deep follicular lesion and food poisoning.

Salmonella spp. which infects a number of animal species (Furowicz and Terzolo, 1975) and *S. typhi*, which causes typhoid fever in humans has also been tested against different plant extract and found to effective. *E. coli* and *K. pneuminae* are found to be resistant against all tested plant extract (Table 3). *K. pneuminae* posses the outer protective covering capsules that help in developing resistance against different plant extract. Similarly these strains are of hospital isolates and now Multiple Drug Resistance (MDR) is the serious problem, so that they might have developed resistance to different plant extract. *P. aeruginosa,* an ESBL producer was found to susceptible by different plant extract *O. sanctum*, *E. caryophyllata*, *A. bidentata* and *A. indica.*

Intensive use of antibiotics often resulted in the development of resistant strains (Sydney et al., 1980), these create a problem in treatment of infectious diseases, furthermore antibiotics sometimes associated with side effects (Cunha, 2001) whereas there are some advantages of using antimicrobial compounds of medicinal plants such as often fewer side effects, better patient tolerance, relatively less expensive, acceptance due to long history of use and being renewable in nature (Vermani and Garg, 2002). Because of this, the search for new antibiotics continues unabated. These findings support the traditional knowledge of local users and it is a preliminary, scientific, validation for the use of these plants for antibacterial activity to promote proper conservation and sustainable use of such plant resources.

Awareness of local community should be enhanced incorporating the traditional knowledge with scientific findings. In conclusion, the results of the present study support the folkloric usage of the studied plants and suggest that some of the plant extracts possess compounds with antimicrobial properties that can be further explored for antimicrobial activity. This antibacterial study of the plant extracts demonstrated that folk medicine can be as effective as modern medicine to combat pathogenic microorganisms. The millenarian use of these plants in folk medicine suggests that they represent an economic and safe alternative to treat infectious diseases.

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