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Effect of various chromatographic terpenoid fractions of *Luffa cylindrica* seeds on in-vitro antimicrobial studies

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SUMMARY

The objective of the present investigation is to evaluate the antimicrobial potency of the terpenoid fractions isolated from *Luffa cylindrica* seeds against various pathogenic microbes. The seeds were powdered and extracted with methanol in soxhlet appratus based on phytochemical screening. Three terpenoid components were isolated by column chromatography and identified by thin layer chromatography and chemical analysis which were designated as $LCSF_4^*$, $LCSF_6^*$ & $LCSF_8^*$ respectively. Disc diffusion method was employed to determine the antimicrobial effectiveness of test compounds I, II and III ($LCSF_4^*$, $LCSF_6^*$ & $LCSF_8^*$) against 6 microbial species viz., *Staphylococcus (S.) aureus, Bacillus (B.) subtilis, Escherichia (E.) coli, Pseudomonas (P.) aeruginosa, Candida (C.) albicans* and *Aspergillus niger*. The disc was saturated with 100 μl of each compound, allowed to dry and introduced on the upper layer of seeded agar plate. The plates were incubated overnight at 37°C. Microbial growth was determined by measuring the zonal inhibition diameters. Compound I showed maximum potency against gram positive *S. aureus* (21 mm) in comparison with standard ciprofloxacin (38 mm), whereas the same compound was completely devoid of activity against both the fungi tested. Compound II was found to be highly sensitive against both the gram negative *E. coli* (20 mm) and *P. aeruginosa* (22 mm). Compound II was found to exhibit maximum potency against the fungi *C. albicans* (15 mm) and *A. niger* (20 mm). Compound III was found to be very effective against both the gram positive *S. aureus* (20 mm) and *B. subtilis* (15 mm) respectively.

Key words: Column isolates; *Luffa cylindrica* seeds; Disc method; Antimicrobial activity; Terpenoids

INTRODUCTION

The use of and search for drugs and dietary supplements derived from plants have accelerated in recent years. While 25 to 50% of current pharmaceuticals are derived from plants, none are used as effective antimicobials. Traditional healers have long used plants to prevent or cure infectious conditions (Cowan, 1999).

Clinical microbiologists have two reasons to be interested in the topic of antimicrobial plant extracts. First, it is very likely that these phytochemicals will find their way in to the arsenal of antimicrobial drugs prescribed by physicians; Several are

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already being tested in humans. It is reported that, on average, two or three antibiotics derived from microorganisms are launched each year (Clark, 1996). After a downturn in that pace in recent decades, the pace is again quickening as Scientists realize that the effective life span of any antibiotic is limited. Worldwide spending on finding new anti-infective agents is expected to increase 60% from the spending levels in 1993 (Alper, 1998). New sources, especially plant sources, are also being investigated. Second, the public is becoming increasingly aware of problems with the over prescription and misuse of traditional antibiotics. Main stream medicine is increasingly receptive to the use of antimicrobial and other drugs derived from plants, as traditional antibiotics becomes ineffective and as new, particularly viral, diseases remain intractable to this type of drug. Another driving factor for the renewed interest in plant microbials in the past 20 years has been the rapid rate of species extinction (Lewis and Elvin-Lewis, 1995).

Plants have an almost limitless ability to synthesize aromatic substances, most of which are secondary metabolites, of which at least 12,000 have been isolated, a number estimated to be less than 10% of the total (Schultes, 1978). In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects and herbivores. With the emergence of new infectious diseases, as well as the emergence of bacterial strains resistant to existing antibiotics, there is an enormous challenge to researchers to develop new methods for treating both existing infectious diseases and those emerging as new health threats (Strohl, 1997).

Luffa cylindrica (Cucurbitaceae) is a common annual herb, growing on hedges and shrubs in garden and coastal areas. The herb is reported to contain a neutral bitter principle and an alkaloid, saponins and flavonoids. Plant is bitter tonic, emetic, diuretic and purgative and useful in asthma, skin diseases and splenic enlargement.

The fruits are anthelmintic, carminative, laxative, expectorant, tonic and galactogogue and are useful in fever, syphilis, tumours, bronchitis, splenopathy and leprosy. The seed oil is used in leprosy and skin diseases (Chopra *et al.*, 2002; Rastogi and Mehrotra, 2004). The extracts of the seeds and leaves of *Luffa cylindrica* has been already reported for antimicrobial activity against *Escherichia (E.) coli, Staphylococcus (S.) aureus, Salmonella typhi and Bacillus (B.) subtilis* (Oyetayo *et al.*, 2007). In view of the widest pharmalogical activity of *Luffa cylindrica*, the present study has been undertaken to investigate the effect of various chromatographic fractionated isolates from the seeds on different strains of clinical pathogenic bacterias and fungi.

MATERIALS AND METHODS

Plant material

The seeds of *Luffa cylindrica* were collected from the road sides of Meerut. The plant material was authenticated by an acknowledged Botanist, Dr. Surabhi Singhal of the Research Department of Microbiology, IIMT College of Medical Sciences, Meerut and the voucher specimen was deposited (IIMT/BD/01/04/2009/02/Tech. 2005).

Phytochemical Extraction

The seeds of *Luffa cylindrica* were dried at room temperature and reduced to a coarse powder. The powdered material was subjected to preliminary phytochemical screening (Mohamed Ali, 1998) for the identification of various phyto constituents (Table 1). Then the powder (113.5 g) was subjected to soxhlet extraction with methanol separately for 72 h at a temperature of 60 - 80 ^{o}C. The total methanol extract (TME) was concentrated and the solvent was completely removed by Rotary Vacuum Evaporator (Buchi). Green waxy residue was obtained, which was stored in dessicator.

Isolation and identification of terpenoid bioactive constituents from TME

The total methanol extract (0.67 g) was taken in a

Table 1. Preliminary Phytochemical Screening of *Luffa cylindrica* Seed Extract

S. No.	Solvents	Alkaloids (Hager's test)	Glycosides (Borntrag-ers test)	Steroids (Salkovaski test)	Terpenoids (Rochan test)	Flavonoids (Shinoda test)	Carbohydrates (Benedict's Test)	Saponins (Foam test)
1.	Petroleum ether	–	–	–	–	–	–	–
2.	Chloroform	++	–	–	–	–	++	–
3.	Benzene	++	–	–	–	–	–	–
4.	η-Butanol	–	–	–	–	–	+	–
5.	Ethanol	–	–	–	–	–	+	–
6.	Ethyl acetate	+	–	–	–	–	–	–
7.	Methanol	–	–	+	++	–	–	–
8.	Water	–	–	–	–	–	–	++

++, Predominant active constituent; +, Moderate active constituent; –, Absence of active constituent.

china dish separately and heated continuously on a water bath by gradually adding dichloromethane in small portion with constant starring till desired consistency was obtained. Silica gel (for column chromatography, 230 – 400 mesh size) was then added twice the amount of extract slowly with continuous mixing till the desired consistency of the mixture obtained. It was then air-dried and larger lumps were broken to get a smooth free flowing mixture.

Two columns of 5.0 feet length and 16 mm of internal diameter were taken and dried. The lower end of the column was plugged with absorbent cotton wool. The column was clamped and fitted in vertical position on a stand. The column was then half filled with hexane. Silica gel was then poured in small portions (13.5 g) and allowed to settle gently until the necessary length of the column was obtained. The dried silica gel slurry containing the total methanol extract of seeds was poured in the column separately and then eluted successively with different solvents, in the order of ethyl acetate: η–hexane (5:9.5), ethyl acetate: η–hexane (1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2), ethyl acetate, ethyl acetate: methanol (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9), methanol, methanol: water (8:2, 6:4, 4:6, 3:7, 2:8) and water.

The fractions collected in the conical flask were marked. The marked fractions were subjected to TLC to check homogeneity of various fractions. Chromatographically identical various fractions (having same R_f values) were combined together and concentrated. They were then crystallized with suitable solvent systems.

Elution of seed drug in column with ethyl acetate-n-hexane (3.5:6.5), i.e. (fraction 4 and 5) yielded slightly greenish yellow amorphous powder, R_f: 0.84 (Ethyl acetate: n-hexane = 60:40) for terpenoids, which was similarly identified with qualitative chemical analysis (Rochan test) and was designated as $LCSF_4^*$.

Similarly elution of seed drug in column with ethyl acetate-n-hexane (5.5:4.5), i.e. (fraction 6 and 7) yielded pale green amorphous powder, R_f:0.98 (n-hexane: Ethyl acetate = 1:1) for terpenoids using antimony (III) chloride in chloroform as visualizing reagent, which was further confirmed with qualitative chemical analysis (Rochan test) and was designated as $LCSF_6^*$.

Elution of seed drug in column with ethyl acetate-n-hexane (7.5:2.5), i.e. (fraction 8 and 9) yielded light green amorphous powder, R_f:0.87 (n-hexane:Ethyl acetate: 1:1) for terpenoids using antimony (III) chloride in chloroform as visualizing reagent, which was further confirmed with qualitative chemical analysis (Rochan test) and was designated as $LCSF_8^*$. Hence the three isolated terpenoid pure components ($LCSF_4^*$;

$LCSF_6^*$ and $LCSF_8^*$) from the seeds of *Luffa cylindrica* were subjected to in-vitro antimicrobial screening against various pathogenic microbes.

Microbial strains, drugs, and chemicals

The investigated microbial strains were obtained from National Chemical Laboratory (NCL), Pune, India. Amongst the studied microbial strains, *S. aureus* ATCC 29737 and *B. subtilis* ATCC 6633 are gram positive bacteria; *E. coli* ATCC 8739 and *P (P.) aeruginosa* ATCC 27853 are gram negative bacteria while *Candida albicans* ATCC 2091 and *Aspergillus niger* ATCC 1015 are fungi and were maintained on agar slants at 12 - 18°C. Prior to testing, they were grown in nutrient agar medium and incubated at 37°C for 48 h followed by frequent subculturing to fresh medium, and were used as test bacteria and fungi.

Standard antibiotics Ciprofloxacin and Fluconazole were obtained as gift samples from Cadilla Pharmaceuticals, Gujarat and I.P.C.A. Laboratories Limited, Mumbai. Sterile discs (5 mm) were procured from Hi-media Laboratories Pvt. Ltd., Mumbai. All the solvents and reagents were of AnalaR grade and are used without purification. Glassware is oven or flame dried prior to use. Seed extracts are fractionated by Flash column chromatography using 230 – 400 mesh silica gel supplied by Acme Chemicals Limited, Mumbai, India. The isolated pure components are designated as test compounds I, II and III respectively as $LCSF_4^*$, $LCSF_6^*$ and $LCSF_8^*$.

Antibacterial screening (Paech and Tracey, 1955; Nagarajan *et al.,* 2006).

The testing of antimicrobial activity of test compounds were carried out in-vitro by Kirby-Bauer disc diffusion technique (Bauer *et al.*, 1996). The disc-diffusion method is highly effective for rapidly growing microorganisms and the activities of the test compounds are expressed by measuring the diameter of the zone of inhibition (Hossain *et al.*, 2004). The formation of inhibition zones represents the dynamic interaction between antibiotic diffusion and bacterial growth (Greenwood, 2000). Generally, the more susceptible the organisms, the bigger the zone of inhibition. The method is essentially a qualitative or semi quantitative test, indicating sensitivity or resistance of micro organisms to the test materials as well as bacteriostatic or bactericidal activity of a compound (Reiner, 1982).

The antibacterial activity of *Luffa cylindrica* seed isolates $LCSF_4^*$, $LCSF_6^*$ and $LCSF_8^*$ were determined against two gram-positive (*S. aureus* and *B. subtilis*) and two gram-negative (*E coli* and *aeruginosa*) bacteria. Mueller Hinton Agar No. 2 was used as an assay medium. Inoculum size was maintained as 1×10^8 cells/ml. The media and test bacterial cultures were poured into petridishes (Hi-media). The test strain (200 µl) was inoculated into the media when the temperature reached 40 – 42°C. The test compounds $LCSF_4^*$, $LCSF_6^*$ and $LCSF_8^*$ (each 100 µl), which was previously dissolved in Dimethyl formamide (Ebner *et al.*, 2008) were impregnated into sterile discs (5 mm) and then allowed to dry. The disc was then introduced into medium with the bacteria. The plates were incubated overnight at 37°C. Microbial growth was determined by measuring the diameter of zone of inhibition in milimeters and its size was compared to that contained in a standardized chart (Cappuccino and Sherman, 1996). Based on this comparison, the test organism was determined to be resistant, intermediate, or susceptible to the antibiotic. The diameters of the zones of inhibition produced by the compounds were also compared with the standard antibiotic (Ciprofloxacin 5 µg/disc).

The experiments were performed three times to minimize error and the mean values are presented in Table 2.

Antifungal assay

The same set of test compounds ($LCSF_4^*$, $LCSF_6^*$ and $LCSF_8^*$) were also tested for antifungal activity against *Candida albicans* and *Aspergillus niger* by paper disc diffusion method (Seely and Van Denmark,

Table 2. Antimicrobial Activity of Methanolic Extract Isolates from the Seeds of *Luffa cylindrica*

S. No.	Organisms tested	Strain number	Concentration of the test sample	Diameter of Zone of Inhibition (mm)			
				$LCSF_4^*$ (Test Compd.I)	$LCSF_6^*$ (Test Compd.II)	$LCSF_8^*$ (Test Compd. III)	Standard Drugs Ciprofloxacin (CIPS)/ Fluconazole (FLU)
1.	*S. aureus*	ATCC 29737	100 µg/ml	21	16	20	38 (CIPS)
2.	*Bacillus subtilis*	ATCC 6633	100 µg/ml	NS	13	15	45 (CIPS)
3.	*E. coli*	ATCC 8739	100 µg/ml	18	20	18	42 (CIPS)
4.	*P. aeruginosa*	ATCC 25619	100 µg/ml	20	22	18	40 (CIPS)
5.	*Candida albicans*	ATCC 2091	100 µg/ml	NS	15	10	22 (FLU)
6.	*Aspergillus niger*	ATCC 1015	100 µg/ml	NS	20	18	28 (FLU)

ATCC–American Type Culture Collection, **$LCSF_4^*$–***Luffa cylindrica*** Seed Fraction–4&5, **$LCSF_6^*$–***Luffa cylindrica* Seed Fraction–6&7, **$LCSF_8^*$–***Luffa cylindrica* Seed Fraction–8&9.

1972). These organisms were maintained on Sabouraud's agar slants by regular sub-culturing. *Candida albicans* was cultured every week and the rest of organism at an interval of twelve days. The test compounds were assayed for antifungal activity in-vitro by using the liquid media described by E. Joan Stokes *et al.*, 1975. The final pH of the medium was adjusted to 5.2. Fluconazole (10 µg/disc) was used as a standard drug for control and also tubes without any drug were kept for comparison. The growth or absence of growth was noted visually after seven days of incubation for these organisms and the diameter of zone of inhibition measured using digital vernier calipers. The experiment was done thrice and the mean values are represented in Table 2.

RESULTS

The seeds of *Luffa cylindrica* were powdered and extracted with methanol using Soxhlet apparatus based on the preliminary phytochemical screening (Table 1). Terpenoid components were isolated from the methanol extract by running flash column chromatography viz., $LCSF_4^*$, $LCSF_6^*$ and $LCSF_8^*$ and were further confirmed by TLC and chemical analysis.

Later on, these isolated pure components were subjected to antimicrobial screening using two gram positive bacteria and two gram negative bacteria with two fungi as the micro organisms. The maximum diameter of zone of inhibition in the disc indicated the maximum potency of antimicrobial agent and the results of antimicrobial assay were expressed in Table 2.

Test compound I, $LCSF_4^*$ shows maximum potency against gram-positive *Staph. aureus* with a zonal diameter 21 mm (Standard 38 mm). The above test compound is also effective against gram-negative *P. aeruginosa* (20 mm) and *E. coli* (18 mm), whereas $LCSF_4^*$ was completely devoid of activity against *Bacillus subtilis* and the fungi, *Aspergillus niger* and *Candida albicans*.

Test compound II, $LCSF_6^*$ showed good potency against *P. aeruginosa* (22 mm), *E. coli* (20 mm) and *Aspergillus niger* (20 mm).

As evident from Table 2, Test compound III ($LCSF_8^*$) was found to be highly effective against *S. aureus* (20 mm), *E. coli* (18 mm), *P. aeruginosa* (18 mm) and *A. niger* (18 mm).

Among the isolated pure components tested, Test compound III ($LCSF_8^*$) was found to be very effective against the gram positive organisms tested. As far as the gram negative organisms are concerned, only the Test compound II, $LCSF_6^*$ exhibits maximum potency against E. coli (20 mm) and *P. aeruginosa* (22 mm).

Among the components tested for antifungal

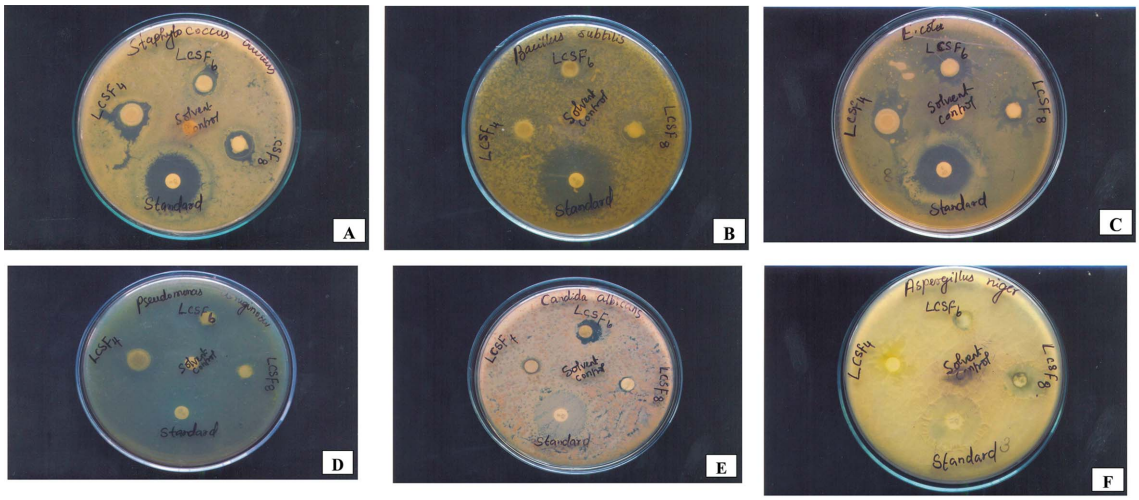

Fig. 1. The antimicrobial activity of pure isolates of *Luffa cylindrica* ($LCSF_4^*$, $LCSF_6^*$ and $LCSF_8^*$) against the different gram-positive and gram-negative bacteria and fungi. **(A)** The disk represents the antibacterial activity with their zone of inhibition of the three test compounds I ($LCSF_4^*$), II ($LCSF_6^*$) and III ($LCSF_8^*$) against *S. aureus* in comparison to the standard ciprofloxacin (± 38 mm). **(B)** The disk represents the antibacterial activity with their zone of inhibition of the three test compounds I ($LCSF_4^*$), II ($LCSF_6^*$) and III ($LCSF_8^*$) against *B. subtilis* in comparison to the standard ciprofloxacin (± 45 mm). **(C)** The disk represents the antibacterial activity with their zone of inhibition of the three test compounds I ($LCSF_4^*$), II ($LCSF_6^*$) and III ($LCSF_8^*$) against *E. coli* in comparison to the standard ciprofloxacin (± 42 mm). **(D)** The disk represents the antibacterial activity with their zone of inhibition of the three test compounds I ($LCSF_4^*$), II ($LCSF_6^*$) and III ($LCSF_8^*$) against P. *aeruginosa* in comparison to the standard ciprofloxacin (± 40 mm). **(E)** The disk represents the antifungal activity with their zone of inhibition of the three test compounds I ($LCSF_4^*$), II ($LCSF_6^*$) and III ($LCSF_8^*$) against *C. albicans* in comparison to the standard Fluconazole (± 22 mm). **(F)** The disk represents the antifungal activity with their zone of inhibition of the three test compounds I ($LCSF_4^*$), II ($LCSF_6^*$) and III ($LCSF_8^*$) against *A. niger* in comparison to the standard Fluconazole (± 28 mm).

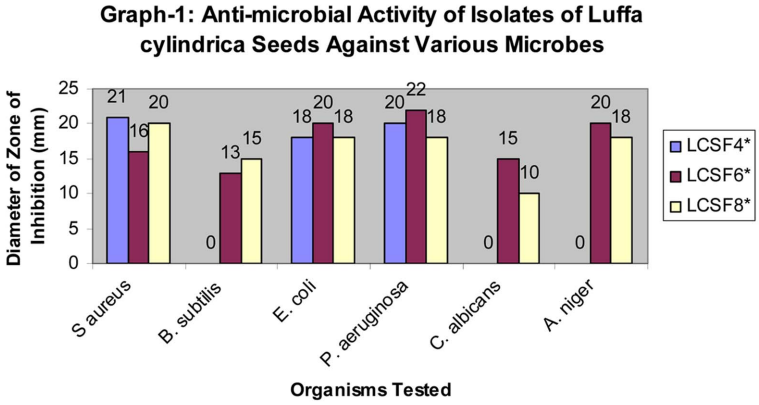

Fig. 2. Anti-microbial activity of isolates of Luffacylindrica seeds against various microbes.

activity, Test compound II ($LCSF_6^*$) was found to exhibit maximum potency against both the fungi *Candida albicans* and *Aspergillus niger* with their respective zone of inhibition, 15 mm and 20 mm. It was concluded that the test compound I ($LCSF_4^*$) doesn't possess antifungal activity at all. All the tested components activity against different microbial strains are graphically represented in Fig. 2.

DISCUSSION

The increasing prevalence of multi drug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial infections and adds urgency to the search for new infection fighting strategies and new effective therapeutic agents (Chanda and Parekh, 2006).

Amongst the 3 isolated components, $LCSF_8^*$ showed good potency against both the gram positive organisms tested with a zonal inhibition diameter of 20 mm and 15 mm (*S. aureus; B. subtilis*), whereas $LCSF_6^*$ showed good potency against gram negative *E. coli* and *P. aeruginosa* (20 mm; 22 mm). The plant extract isolates inhibited the gram negative micro organisms better than gram positive micro organisms.

The maximum antifungal activity was exhibited by methanolic fractions 6 & 7 of *Luffa cylindrica* ($LCSF_6^*$) with their corresponding zone of inhibition (15 mm; 20 mm) against *Candida albicans* and *Aspergillus niger*.

Successful prediction of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. The traditional healers or practitioners make use of water primarily as a solvent, but methanol extracts of these plants are certainly much better and more powerful. This shows that the active components are better soluble in organic solvent (De Boer *et al.,* 2005). These observations can be rationalized in terms of the polarity of the compounds being extracted by each solvent and, in addition to their intrinsic bioactivity, by their ability to dissolve or diffuse in the different media used in the assay. The growth media also seem to play an important role in the determination of the antibacterial activity. Lin *et al.,* 1999 has reported that Mueller-Hinton agar appears to be the best medium to explicate the antibacterial activity and the same was used in the present study.

Preliminary identification of the test compounds I ($LCSF_4^*$), II ($LCSF_6^*$) and III ($LCSF_8^*$) reveals the presence of terpenoids by TLC and chemical tests after elution from the column. Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen substituted derivatives (Geissman, 1963). The fragrance of plants is carried in the so called quinta essentia, or essential oil fraction. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure. They are called terpenes, and when the compounds contain additional elements, usually oxygen, they are termed terpenoids. Terpenenes or terpenoids are active against bacteria (Kubo *et al*., 1992; Habtemariam *et al.,* 1993) and fungi (Taylor *et al*., 1996; Rana *et al*., 1997). Since our test components contain terpenoid as predominant active constituent, they inhibit the bacterial and fungal strains probably by membrane disruption phenomenon (Cichewicz and Thorpe, 1996) of lipophilic compound as similar to compounds like capsaicin. Accordingly Mendoza *et al.* (1997) have found that increasing the hydrophilicity of Kaurene diterpenoids by addition of a methyl group drastically reduced their antimicrobial activity.

It would be advantageous to standardize methods of extraction and in-vivo testing so that the search could be more systematic and interpretation of results would be facilitated. Also, alternative mechanisms of infection prevention and treatment should be included in initial activity screenings. Disruption of adhesion is one example of an anti-infection activity not commonly screened for currently. Attention to these issues could usher in a badly needed new era of chemotherapeutic treatment of infection by using plant derived principles. The antimicrobial property of the blended of these terpenoid fractions may need to study in future to establish an even more comprehensive performance for these fractions.

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