

Activity-Directed Fractionation & Isolation of Three Antimicrobial Compounds from *Abrus Precatorius L.* Seeds

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Shortened Title: Anti-microbial activity of Isolates of *Abrus precatorius*.

Summary

The objective of the present investigation is to evaluate the antimicrobial potency of the various chromatographic fractions isolated from *Abrus precatorius* seeds against various pathogenic microbes. The seeds were powdered and extracted with methanol in soxhlet apparatus based on phytochemical screening. Three pure components were isolated by column chromatography and identified by thin layer chromatography and chemical analysis which were designated as F₁₀, F₁₁^{*} & F₁₄ respectively.

Disc diffusion method was employed to determine the antimicrobial effectiveness of test compounds I, II and III (F₁₀, F₁₁^{*} & F₁₄) against 6 microbial species viz., *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida 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 *B. subtilis* (18 mm) in comparison with standard ciprofloxacin (26 mm), whereas the same compound was devoid of activity against the fungi *A. niger* (3 mm) & gram negative *P. aeruginosa* (2 mm) tested. Compound II was found to be sensitive against both the gram positive *B. subtilis* (16 mm) and the fungi *C. albicans* (14 mm). Compound III was found to exhibit maximum potency against the fungi *C. albicans* (23 mm) and gram positive *S. aureus* (15 mm). Among the 3 isolated components, Compound III was found to be very effective against one of the gram positive *S. aureus* and gram negative *E.coli* and all the fugal strains used.

Key words: Column isolates, *Abrus precatorius* seeds, disc method, antimicrobial activity.

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 antimicrobials. Traditional healers have long used plants to prevent or cure infectious conditions (1).

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 already being tested in humans. It is reported that, on average, two or three antibiotics derived from microorganisms are launched each year (2). 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 (3). 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 (4).

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 (5). 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 (6).

Abrus precatorius (Fabaceae) is found through the plains of India, from the Himalayas down to Southern India and Ceylon. Seeds are purgative, emetic, tonic, antiphlogistic, aphrodisiac and anti-ophthalmic (7).

Alcoholic extract of seeds showed parasympathomimetic effect on smooth muscle of guinea pig and rabbit and skeletal muscle of frog. The aqueous extract showed anthelmintic activity which was absent in extract prepared by boiling seeds in water (8). The extracts of the seeds of *Abrus precatorius* has been already reported for antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* by agar well diffusion technique (9). In view of the widest pharmacological activity of *Abrus precatorius*, the present study has been undertaken to investigate the effect of various chromatographic fractionated isolates from the seeds on different strains of clinical pathogenic bacteria and fungi by employing disc diffusion technique in order to fulfill the method optimization strategy of herbal drug standardization and harmonization.

Materials and Methods

Plant Material

The seeds of *Abrus precatorius* 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/01/05/2009/02/Tech. 2005).

Phytochemical Extraction

The seeds of *Abrus precatorius* were dried at room temperature and reduced to a coarse powder. The powdered material was subjected to preliminary phytochemical screening (10) for the identification of various phyto constituents (Table 1). Then the powder (113.5 gm) was subjected to soxhlet extraction with methanol separately for 72 hours at a temperature of 60-80° 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.

Table 1. Preliminary Phytochemical Screening of *Abrus precatorius* Seed Extract

S. No.	Solvents	Alkaloids (Hager's test)	Glycosides (Borntragers 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.	n- Hexane	-	-	-	-	-	++	-

++ Predominant active constituent

+ Moderate active constituent

- Absence of active constituent

Isolation and Identification of Bioactive Constituents from TME

The total methanol extract (0.67 gm) was taken in a china dish separately and heated continuously on a water bath by gradually adding dichloromethane in small portion with constant stirring 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 ft. 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 gm) 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), Chloroform: methanol (2:8, 4:6, 6:4, 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 chloroform-methanol (6:4), i.e. (fraction 10) yielded slightly greenish yellow amorphous powder, R_f : 0.86 (Benzene: chloroform: 60:40) for flavonoids, which was similarly identified with qualitative chemical analysis (Shinoda test) and was designated as F_{10} .

Similarly elution of seed drug in column with chloroform-methanol (4:6), i.e. (fraction 11 and 12) yielded pale green amorphous powder, R_f : 0.49 (Chloroform: methanol: 2:8) for flavonoids using UV and iodine chamber, which was further confirmed with qualitative chemical analysis (Shinoda test) and was designated as F_{11}^* .

Elution of seed drug in column with ethyl acetate-methanol (1:9), i.e. (fraction 14) yielded light green amorphous powder, R_f : 0.98 (methanol: Ethyl acetate: 9:1) for glycosides using UV and iodine chamber, which was further confirmed with qualitative chemical analysis (Borntrager's test) and was designated as F_{14} . Hence the three isolated pure components (F_{10} ; F_{11}^* and F_{14}) from the seeds of *Abrus precatorius* were subjected to *in-vitro* antimicrobial screening against various pathogenic microbes.

Materials

The investigated microbial strains were obtained from National Chemical Laboratory (NCL), Pune, India. Amongst the studied microbial strains, *Staphylococcus aureus* ATCC 29737 and *Bacillus subtilis* ATCC 6633 are gram positive bacteria; *Escherichia coli* ATCC 8739 and *Pseudomonas 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 hours 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 F_{10} ; F_{11}^* and F_{14} .

Antibacterial Screening (11-12)

The testing of antimicrobial activity of test compounds were carried out *in-vitro* by Kirby-Bauer disc diffusion technique (13). 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 (14). The formation of inhibition zones represents the dynamic interaction between antibiotic diffusion and bacterial growth (15). 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 (16).

The antibacterial activity of *Abrus precatorius* seed isolates F₁₀; F₁₁^{*} and F₁₄ were determined against two gram-positive (*S. aureus* and *B. subtilis*) and two gram-negative (*E. coli* and *P. aeruginosa*) bacteria. Mueller Hinton Agar No. 2 was used as an assay medium. Inoculum size was maintained as 1×10⁸ 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 F₁₀; F₁₁^{*} and F₁₄ (each 100 µl), which was previously dissolved in Dimethyl formamide (17) 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 millimeters and its size was compared to that contained in a standardized chart (18). 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 (F₁₀; F₁₁^{*} and F₁₄) were also tested for antifungal activity against *Candida albicans* and *Aspergillus niger* by paper disc diffusion method (19). 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 (20). 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 *Abrus precatorius* were powdered and extracted with methanol using Soxhlet apparatus based on the preliminary phytochemical screening (Table 1). Pure components were isolated from the methanol extract by running flash column chromatography viz., F₁₀; F₁₁^{*} and F₁₄ 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.

Table 2. Antimicrobial Activity of Methanolic Extract Isolates from the Seeds of *Abrus precatorius*.

S.No.	Organisms Tested	Strain Number	Concentration of the Test Sample	Diameter of Zone of Inhibition (mm)			
				F ₁₀ (Test Compd.I)	F ₁₁ [*] (Test Compd.II)	F ₁₄ (Test Compd. III)	Standard Drugs Ciprofloxacin (CIPS)/ Fluconazole (FLU)
1.	<i>Staphylococcus aureus</i>	ATCC 29737	100 µg/ml	9	6	15	32 (CIPS)
2.	<i>Bacillus subtilis</i>	ATCC 6633	100 µg/ml	18	16	12	26 (CIPS)
3.	<i>Escherichia coli</i>	ATCC 8739	100 µg/ml	7	5	13	38 (CIPS)
4.	<i>Pseudomonas aeruginosa</i>	ATCC 25619	100 µg/ml	2	4	1	40 (CIPS)
5.	<i>Candida albicans</i>	ATCC 2091	100 µg/ml	11	14	23	23 (FLU)
6.	<i>Aspergillus niger</i>	ATCC 1015	100 µg/ml	3	11	13	25 (FLU)

ATCC– American Type Culture Collection

F₁₁^{*} – *Abrus precatorius* Seed Fraction–11&12F₁₀– *Abrus precatorius* Seed Fraction–10F₁₄– *Abrus precatorius* Seed Fraction–14

The antimicrobial activity of pure isolates of *Abrus precatorius* (F₁₀, F₁₁^{*} and F₁₄) against the different gram-positive and gram-negative bacteria and fungi is demonstrated very well in Figures A to F.



Fig. A: The disk represents the antibacterial activity with their zone of inhibition of the three test compounds I (F₁₀), II (F₁₁^{*}) and III (F₁₄) against *S. aureus* in comparison to the standard ciprofloxacin (\pm 32 mm).

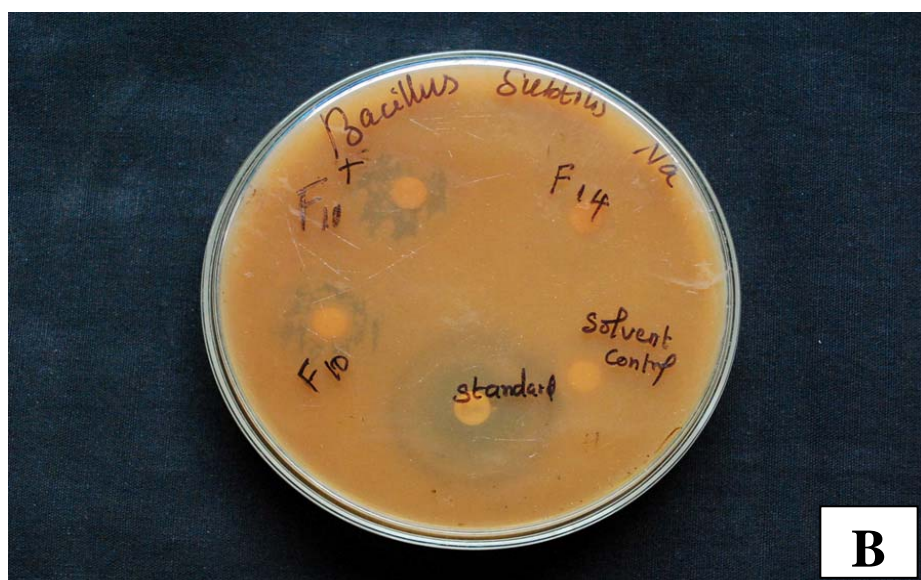


Fig. B: The disk represents the antibacterial activity with their zone of inhibition of the three test compounds I (F₁₀), II (F₁₁^{*}) and III (F₁₄) against *B. subtilis* in comparison to the standard ciprofloxacin (\pm 26 mm).



Fig. C: The disk represents the antibacterial activity with their zone of inhibition of the three test compounds I (F_{10}), II (F_{11}^*) and III (F_{14}) against *E. coli* in comparison to the standard ciprofloxacin (± 38 mm).

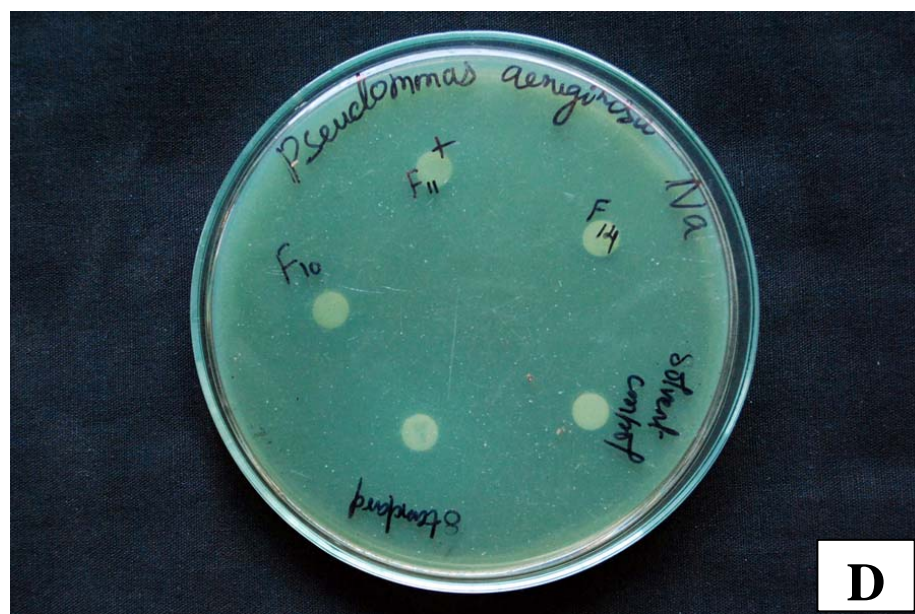


Fig. D: The disk represents the antibacterial activity with their zone of inhibition of the three test compounds I (F_{10}), II (F_{11}^*) and III (F_{14}) against *P. aeruginosa* in comparison to the standard ciprofloxacin (± 40 mm).



Fig. E: The disk represents the antifungal activity with their zone of inhibition of the three test compounds I (F₁₀), II (F₁₁^{*}) and III (F₁₄) against *C. albicans* in comparison to the standard Fluconazole (± 23 mm).



Fig. F: The disk represents the antifungal activity with their zone of inhibition of the three test compounds I (F₁₀), II (F₁₁^{*}) and III (F₁₄) against *A. niger* in comparison to the standard Fluconazole (± 25 mm).

Test compound I, F₁₀ shows maximum potency against gram-positive *Bacillus subtilis* with a zonal diameter 18 mm (Standard 26 mm). The above test compound is also moderately effective against gram-positive *Staph. aureus* (9 mm) and the fungi *Candida albicans* (11 mm), whereas F₁₀ was found to be resistant against *Pseudomonas aeruginosa* (2 mm) and the fungi, *Aspergillus niger* (3 mm).

Test compound II, F₁₁^{*} showed good potency against *B. subtilis* (16 mm) and *C. albicans* (14 mm), whereas the same test compound was found to be moderately sensitive against *Aspergillus niger* (11 mm).

As evident from Table 2, Test compound III (F₁₄) was found to be highly effective against *C. albicans* (23 mm) in comparison with the standard ciprofloxacin (23 mm). The same compound was effective against *S. aureus* (15 mm), *E. coli* (13 mm) and *A. niger* (13 mm) respectively.

Among the isolated pure components tested, Test compound III (F₁₄) was found to be very effective against the gram positive organisms and gram negative organisms tested, being only with the exception *P. aeruginosa* (1 mm). As far as the gram negative organisms are concerned, only the Test compound III, F₁₄ exhibits considerable potency against *E. coli* (13 mm) and none of the isolated test compounds was effective against *Pseudomonas aeruginosa*.

Among the components tested for antifungal activity, Test compound III (F₁₄) & Test compound II (F₁₁^{*}) was found to exhibit maximum potency against both the fungi *Candida albicans* and *Aspergillus niger* with their respective zones of inhibition, 23 mm; 13mm and 14 mm; 11 mm. It was concluded that none of the test compounds were found to be effective against opportunistic pathogen *Pseudomonas aeruginosa* and the test compound III was found to have comparatively equipotent activity similar to standard ciprofloxacin against the fungi *Candida albicans* (23 mm each).

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 (21).

Amongst the 3 isolated components, F₁₄ showed good potency against both the gram positive organisms tested with a zonal inhibition diameter of 15 mm and 12 mm (*S. aureus*; *B. subtilis*), whereas F₁₄ showed good potency against gram negative *E. coli* alone (13 mm). The plant extract isolates inhibited the gram positive micro organisms better than gram negative micro organisms.

The maximum antifungal activity was exhibited by methanolic fractions 14 of *Abrus precatorius* (F₁₄) with their corresponding zone of inhibition (23 mm; 13 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 (22). 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. It has been already reported that Mueller-Hinton agar appears to be the best medium to explicate the antibacterial activity (23) and the same was used in the present study.

Preliminary identification of the test compounds I (F₁₀), II (F₁₁^{*}) and III (F₁₄) reveals the presence of flavonoids (for test compounds I & II) and glycosides by TLC and chemical tests after elution from the column. The best-described property of almost every group of flavonoids is their capacity to act as antioxidants. The flavones and catechins seem to be the most powerful flavonoids for protecting the body against reactive oxygen species. Body cells and tissues are continuously threatened by the damage caused by free radicals and reactive oxygen species, which are produced during normal oxygen metabolism or are induced by exogenous damage (24-25). The mechanisms and the sequence of events by which free radicals interfere with cellular functions seem to be lipid peroxidation, which results in cellular membrane damage. This cellular damage causes a shift in the net charge of the cell, changing the osmotic pressure, leading to swelling and eventually cell death. The activity of quercetin, for example, has been at least partially attributed to inhibition of DNA gyrase. It has also been proposed that sophoraflavone G and (-)-epigallocatechin gallate inhibit cytoplasmic membrane function, and that licochalcones A and C inhibit energy metabolism (26).

Since our test compounds I & II contain flavonoid as predominant active constituent, they inhibit the bacterial and fungal strains probably by inhibiting cytoplasmic membrane function. The antifungal activity of glycosides of test compound III is generally attributed to membrane-permeabilizing properties. The precise mechanism of membrane disruption is unknown, but the sugars are critical for activity (27-29). Hence the activities of results obtained by disc diffusion technique are comparatively better than the well diffusion technique, which fulfills the antimicrobial method optimization of herbal drug standardization and harmonization.

It would be again 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 work in this respect is in progress.

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