Journal Of Harmonized Research (JOHR)

Journal Of Harmonized Research in Pharmacy
4(2), 2015, 148-161

ISSN 2321 – 0958

Original Research Article

CHEMICAL *FINGERPRINT ANALYSIS, IN-VITRO* ANTIMICROBIAL ACTIVITY AND TOXICOLOGICAL ASPECTS OF A POLYHERBO-MINERAL OIL FORMULATION: *KUSTHARAKSASA TAILA*

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Abstract: The objectives of the study were to test the polyherbo-mineral oil *Kustharaksasa taila* (KT) for *in vitro* antimicrobial activity against Gram-negative and positive bacteria, *Candida* species and dermatophytes, to evaluate its acute toxicological effects *in vivo* and to obtain the chemical fingerprints using various analytical techniques.The antimicrobial activity, in vitro, was initially evaluated by the agar-well diffusion technique, and the MIC and minimum fungicidal concentration (MFC) were determined by the broth micro-dilution method. The acute toxicological effects were determined in rabbits and rats, respectively. The chemical fingerprints of the oil were obtained by gas chromatography coupled to mass spectroscopy, High performance thin layer chromatography (HPTLC), Fourier transform infra red (FTIR) spectroscopy and X-Ray powder diffraction (XRPD) analysis.The KT was found effective against all tested strains in agar well diffusion method. The minimum inhibitory concentration of KT ranged from 19.5 to 312 µg/ml, the minimum bactericidal concentration ranged from 39 to 312µg/ml while the minimum fungicidal concentration ranged from 156 to 625 µg/ml. The acute administration and dermal application of KT was devoid of toxicity in rats. The characteristic chromatographic peaks of the chromatographic fingerprints and d spacing values in the XRD spectra were summarized for future qualitative analysis. KT may be a promising source in the search for new antimicrobial drugs due to its efficacy and low toxicity.

Key words: Ayurveda, antibacterial, antidermatophytic

Introduction: Skin diseases like ringworm, leprosy, leucoderma and eczema are prominent problems in developed countries of tropical and subtropical region. Bacterial and fungal strains like *Candida*, *Microsporum*, and *Trycophytons* are the most common pathogens in human and animals. There has been constant search for biologically active compounds from natural sources due to a lack of efficacy, side effects and resistance offered by microorganisms to the existing drugs (Fontenelle *et al.*, 2007;

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Received on: May 2015
Accepted after revision: May 2015

Sanguinetti *et al.*, 2007; Jardim *et al*., 2008). Further, most antifungal and anti bacterial creams used are expensive. Therefore topical, traditional medicines, especially *taila* (a medicinal oil) preparations using local herbal material are very popular (Burdock 2008, Cross et al, 2008).

Taila are polyherbal oil preparations. They form an important class of Ayurvedic medicinal system. They were formulated with the main objective of incorporating the fat-soluble fractions of herbal drugs to a suitable oil base. There are number of marketed Ayurvedic *taila* which are used for treatment of skin diseases (Mukerjee 2002, Anonymous 1978). With an increasing number of people switching once again towards the alternative system of medicine, it is important to ensure that they get authentic medicines of recommended quality (Trease and Evan 2002, Fontenelle *et al*., 2007, Salman *et al*. (2008).

The chemical fingerprints obtained by chromatographic and electrophoretic techniques are strongly recommended for the purpose of quality control. Potential utility of XRPD as a fingerprinting tool for characterization of mineral components in traditional medicines is well recognized (EMEA, 2007; Kyono *et al*., 2005). Biological investigations using *in-vivo* and *in- vitro* models are conducted to evaluate the claimed therapeutic effect of herbal medicine (Filon *et al*., 2009). *In-vitro* antimicrobial techniques like agar well diffusion technique and broth micro dilution test are most widely used models to screen antibacterial and antifungal activity of a topical medicinal preparation (Hammer *et al*., 2002; Kan *et al*., 2006; Salman *et al*., 2008). Toxicological investigations are required to supplement human experience in defining possible toxicity from short-term use, but are particularly important in detecting toxicity that may occur either after prolonged exposure or years after the exposure has been discontinued.

Many authors have generated figurative data to provide scientific basis to the use of medicinal oils as promising antimicrobial agents. Antimicrobial and toxicology of *Tuvaraka taila* was reported by Dubey *et al.* (2010). Chemical composition, toxicological aspects and antifungal activity of essential oil (EO) from *Lippia sidoides* Cham. was reported by Fontenelle *et al.* (2007). *In vitro* activity of *Citrus bergamia* (bergamot) EO against clinical isolates of dermatophytes was reported by Sanguinetti *et al.* (2007). Kan *et al.* (2006) reported chemical composition and antibacterial activity of *Satureja cuneifolia* EO. Antifungal activity and composition of EO of Brazilian *Chenopodium* was reported by Jardim *et al.* (2008). Salman *et al.* (2008) reported antimicrobial activity of *Nigellia sativa* seed oil. *In vitro* activity of tea tree EO against dermatophytes and filamentous fungi was carried out by Hammer *et al.* (2002).

Kustharaksasa taila (KT) is widely marketed Ayurvedic polyherbal oil used for various skin aliments like leucoderma, psoriasis, bacterial and fungal infections. *Kustharaksasa* taila is a polyherbo-mineral oil containing powdered mercury, sulphur (as mercuric sulphide), cinnabar (HgO), realgar (As_4S_4), fine copper powder, orpiment (As_2S_3), *Allium sativum* bulb (family, Alliaceae), *Plumbago zylenicum* root (family, Plumbaginaceae), *Cassia fistula* (family, Leguminosae), *Alstonia scholaris (*family, Apocynaceae), *Psoralea Corylifolia(*family, Fabaceae) and *Saussurea lappa* root (family, Asteraceae), digested in mustard seed oil(*Brassica nigra.* Family, Brassicaceae) as per classical Ayurvedic text (Sharma, 1985; Anonymous, 2000). The components of KT are reported to have wide range of antimicrobial activity (Arulmozhi *et al*. 2007; Schmidtke *et al.* (2006); Benkeblia, (2004); Ohta *et al.*, 1999; Prasad *et al*., 2004; Ahmad *et al*. (1998)). But no scientific data on qualitative batch to batch consistency, safety and efficacy of KT is available. In the present studies attempt was made to scientifically evaluate the *in vitro* antimicrobial activity of KT. Acute toxicity study was performed for KT

and chemical fingerprints were obtained using various analytical techniques.

Material and Methods

Test material

Commercial marketed poly herbal oil formulation '*Kustharaksasa taila'* manufactured by three different reputed Ayurvedic drug manufacturers was collected in triplicate from retail pharmacies in Indore, Madhya Pradesh, India.

Chemicals

Gentamycin and Itraconazole were supplied as free samples by Zyg Pharmaceuticals Ltd, Indore Madhya Pradesh, India. Potato dextrose agar, Mueller–Hinton agar and Mueller–Hinton broth were purchased from Merck (Germany), and RPMI 1640 medium from Sigma (USA). Analytical grade solvents were obtained from Merck (Mumbai, India).

Test organisms

Bacterial and fungal strains selected for studies were obtained from the collection of Department of Microbiology (Choitthram Hospital and Medical Research Centre), Indore (Madhya Pradesh) India. The test microorganisms used for the antimicrobial activity screening were Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228 and *Bacillus subtilis* ATCC 6633); Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Kebsiellia spp* (clinical isolate) and *Pseudomonas aeruginosa* ATCC 27853) and dermatophytes (fungal) strains (*Candida albicans* ATCC 10231, *Candida tropicalis* ATCC 20336, *Trichophyton rubrum* ATCC 40051, *Trichophyton* mentagrophytes ATCC 9533, *Microsporum canis* ATCC 32903 and *Epidermophyton floccosum* ATCC 52066).

Animals

Albino rats of Wistar strain of either sex weighing between 150-200 g and inbred female New Zealand white rabbits weighing between 2.0-2.5 kg were used .They were housed in standard cages at room temperature (26 ± 2°C) and 44–56% relative humidity, under a light/dark cycle of 10/12 h, for 1 week before the experiments. Rats were provided with standard rodent pellet diet (Amrut, India), and water *ad libitum*. Rabbits were provided with standard rabbit feed (Amrut, India), and water *ad libitum*. The animals were deprived of food for 24 hrs before experimentation, but had free access to drinking water. All experiments were performed in the morning. The study was approved by the institutional ethical committee, (465/01/96/CPSCSEA) which follows the guidelines of CPSCEA (Committee for the Purpose of Control and Supervision of Experimental on Animals), which complies with international norms of INSA.

Chemical Finger Print Analysis:

***Sample preparation*:** Oil formulations (5g) were extracted with methanol (1:5, w/v) at 50 ºC for 20 min. Then the mixture was centrifuged at 2000×g for 20 min at 4 ºC and the supernatants were collected. The supernatants were pooled and concentrated under vacuum at 45 ºC and made up to 5ml volume with methanol. The extracts were filtered through 0.45µm filter and subjected to GC-MS and HPTLC analysis. KT as such was used FTIR analysis. *KT* was filtered using Whatman filter paper and solid residue was obtained, this was used for XRPD analysis.

Gas chromatography/mass spectral analysis: Perkin Elmer auto system XL GC+ gas chromatograph with turbomass software equipped with electric ionization (EI) and chemical ionization (CI) quadrapole mass analyzer was used for analysis. The MS scan range was 30-1200 atomic mass units (AMU). The chromatographic column for the analysis was a fused silica WCOT-Fused Silica capillary column (30 m x 0.25 mm i.d.; CP-Sil 5CP, 025 μm). The carrier gas used was helium at a flow rate of 1 mL/min. Samples were analyzed with the column held initially at 100 ◦C for 3 min and then increased to 150 ◦C with a 8 ◦C/min heating ramp and then kept at 150 ◦C for 3 min. Finally, temperature was increased to 250 ◦C with a 15 ◦C/min heating ramp and kept at 250

◦C for 3 min. The injection was performed in split mode (50:1) at 250 ◦C. Mass spectra were obtained by electronic impact at 70 V.

High performance thin layer chromatographic analysis: CAMAG HPTLC system (Switzerland) with a Linomat 5 sample applicator was used for the analysis. The analysis was performed in air-conditioned room maintained at 22º C (±2°C) and 55% (±5%) relative humidity. TLC was performed on precoated silica gel HPTLC aluminum plates $60F_{254}$ (10cm×10 cm, 0.2mm thickness, 5–6µm particle size, E-Merck, Germany). Five µl of the sample solutions were spotted as bands of 6mm width , at least 11.6 mm apart, 8mm from the lower edge and at least 15mm from left and right edge of the plate by spray-on technique using the auto sampler fitted with a 100µl Hamilton syringe. The plates were developed to a distance of 85 mm using toluene–ethyl acetate (7:3, v/v) in a CAMAG twin—trough plate development chamber which was lined with a filter paper and presaturated with 30 ml mobile phase in trough opposite to the plate. The developed plates were dried using warm air of hair dryer and scanned at 254nm. A spectro-densitometer (Scanner 3, CAMAG) equipped with 'win CATS' planar chromatography manager (version 1.3.0) software was used for the densitometry measurements, spectra recording and data processing. Absorption/remission were measurement mode at a scan speed of 20nm/s.

Fourier transform infra-red spectroscopic analysis: KT was scanned using a Thermonicolet IR-200 spectrophotometer with a DTGS detector in the region of 400 to 4000 cm^{-1}. Each spectra was an average of 24 scans of 2 cm^{-1} resolution. Sampling was done using attenuated total reflectance (ATR) assembly with a sample holder of Zn-Se crystal.

X-Ray Powder Diffraction analysis: Diffractograms were obtained by scanning the solid residue on Phillips make X-pert powder diffractometer and 2θ scan was from 10 to 100° using Ni filter Cu K alpha radiation and NaI scintillator.

Antimicrobial testing

1. Preparation of bacterial and fungal inocula

Prior to testing, each bacterial isolate was subcultured overnight at 37°C in Muller Hinton agar slant. The slopes were flooded with 0.85% (w/v) saline and transferred to a sterile tube. The bacterial suspension concentration was set to 10^5 colony forming unit(CFU)/ml using 0.5 McFarland standard turbidity. Fungal isolates were subcultured on a potato dextrose agar (PDA) slant at 28°C for 2-10 days or until good conidiation was produced. For each dermatophytic isolate, a suspension of conidia was prepared in 0.85% (w/v) saline by swabbing the colony surface with a sterile swab. After the settling of the larger particles, conidia concentration of inoculum was set to 10^5 CFU/ml using 0.5 McFarland standard turbidity. 100 µl of the suspension of the tested microorganism containing 10^5 CFU/ml of bacterial, 10^5 CFU/ml of yeast was used for testing.

2. Agar-well diffusion susceptibility test

The antibacterial susceptibility of KT was evaluated by the agar-well diffusion method. Petri dishes with a diameter of 15 cm were prepared with potato dextrose agar for fungal strains and Mueller Hinton agar culture for bacterial strains. Wells (6 mm in diameter) were then cut from the agar. The KT was weighed and diluted in dimethyl formamide to obtain the test concentrations of 25, 50, 75 and 100 mg/ml. Stock solutions of gentamycin (1 mg/ml) and Itraconazole (0.5 mg/ml) were prepared in distilled water and tested as positive controls for bacterial and fungal strains respectively. Each bacterial and fungal suspension was inoculated onto the surface of the agar, from which 0.1 ml of inoculum was immediately and evenly distributed with a Drigalski spatula. These plates, after having been at 4°C for 2 hrs were incubated aerobically at 37°C for 24 hrs for bacteria and at 25°C for 24 hrs for yeast. All Petri dishes were examined for zones of growth

inhibition and the diameters of these zones were measured in mm. Each experiment was repeated at least twice. A negative control was also prepared using the same solvent employed to dissolve the KT. Antimicrobial activity was evaluated by measuring the zone of inhibition against the tested microorganisms. The result recorded for each bioassay was the average of 3 tests. The results were expressed as mean ± SD of the diameter of the growth inhibition zones (mm).

3. Broth micro dilution method

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) or minimum bactericidal concentration (MBC) for KT were determined by the broth microdilution method, MICs were determined using Mueller–Hinton broth for bacterial testing and for fungal testing, 3-(*N*-morpholino)propanesulfonic acid-buffered RPMI 1640 medium was used. The KT was 2-fold diluted in DMF to the highest concentration (5 mg/ml) to be tested. Then serial dilutions were made in a concentration range from 78 to 5000 µg/ml in nutrient broth. Gentamycin (7.8-500 µg/ml) was used as a positive control for bacterial strains and itraconazole (0.03 to 16 µg/ml) was used for fungal strains. The microdilution assay was performed in 96-well microdilution plates. Growth and sterile control wells were included for each isolate tested. In brief, the wells of 96-well plates were dispensed with 95 µl of nutrient broth and 5 µl of the inocula. A 100 µl aliquot from the stock solutions of the KT initially prepared at the concentration of 5000 µg/ml was added to the first wells. Then 100 µl from their serial dilutions was transferred into six consecutive wells. The last well containing nutrient broth without compound and the same amount of inocula with previous wells on each strip was used as a negative control. The final volume in each well was 200 µl. The microplates were incubated at 37°C for 24 hrs for bacterial strains and 28°C for 2-8 days for fungal strains. All isolates were run in duplicate and repeated at least twice. MIC endpoints were read as the lowest concentration of antimicrobial that totally inhibited macroscopically visible growth of the inocula.

Toxicological studies

1. Acute oral toxicity studies

The effects of KT were observed after a single *per oral* administration to rats. Initially the study was carried out with one group consisting of three female animals at 200 mg of active ingredient per kg of body weight. Based on these observations the dose was increased to 2000 mg/kg body weight. All rats were subjected to a gross necropsy examination after fourteen days (OECD 2001).

2. Acute dermal toxicity studies

The effects of KT after a single dermal administration to rats were investigated. TT at a dose of 2000 mg/kg of body weight was administered once dermally on an area of 5 × 6 cm on the dorsal thoracic region of five male and five female rats and the duration of the exposure was 24 h. The animals were subjected to a necropsy including a gross pathological examination after fourteen days (OECD 2001).

3. Dermal irritancy test

The effects of KT following a single application to the intact skin of rabbits were examined. The test substance (1.5 g) was spread on cellulose patches in a size of about 2.5 × 2.5 cm and was applied to the intact skin of each of three female white rabbits. At the end of the exposure period (4 h) the dressings and the patches were removed. The skin was examined for erythema/eschar and oedema as well as for other local alterations at 1, 24, 48 and 72 h after patch removal.

Results

The GC-MS chromatogram of *KT* is given in figure 1 and major peaks are summarized in table 2. Various mobile phases were tried for HPTLC fingerprint analysis of *taila* preparations .The mobile phase of toluene: ethyl acetate produced well resolved band of both polar and non polar components of *taila* (maximum number of components of *taila*);

thus it was selected for chromatographic fingerprinting. The chromatographic fingerprinting was reproducible. Total 13 bands were observed in the HPTLC finger print of *KT taila*. The R_f value and area of peaks obtain in fingerprint chromatogram of *KT* are summarized in table 2 and figure 2 .The FTIR spectrum of *Vranaraksasa taila* showed all the characteristic peaks (Figure 3) of mustard oil as summarized in table 3. These quanlitative standards for *taila* preparation are required to ensure safety and efficacy of taila preparation.

Kustharaksasa Taila (KT) contains six mineral components. The characteristic XRD peaks for the mineral components in KT are as follows. Cinnabar [HgS] has characteristic XRD peaks at [2θ] of 31, 54.56 and 72.18 with d-spacing 2.88 Å, 1.68 Å, and 1.30 Å. Realgar [As_4S_4] has characteristic XRD peaks at [2θ] of 32.67, 65.23 and 48.95 with d-spacing 2.73 Å, 1.43 Å and 1.85 Å. Orpiment [As_2S_3] produces characteristic XRD peaks at [2θ] of 18.33, 36.52 and 88.47 with d-spacing 4.83Å, 2.45Å and 1.10Å. Fine copper powder produces characteristic XRD peaks at [2θ] of 43.60 and 49.97 with d-spacing 2.07Å and 1.82Å. The characteristic XRD peaks for mercuric sulphide are at [2θ] of 26.31, 27.67and 42.73 with d-spacing values of 3.38Å, 3.22Å and 2.11Å. XRPD pattern (Figure 4 and Table 4) of all mineral components in *taila*) were characterized for their d-spacing and 2θ values as shown in table (Ramsdell, 1925; Kyono *et al.*, 2005; Morimoto, 1954).The results indicated that in *KT* there is slight deviation of peak intensity and 2 θ values of above characteristic peaks of the mineral components which indicate the possibility of small changes in chemical environment, but d spacing [Å] for the component are unchanged.

The semi-quantative agar well diffusion method is easy to perform, and is useful to detect the activity of antimicrobial agents on large numbers of cultures. It also gives an opportunity to evaluate the antibacterial activity to some extent without performing a MIC assay. The KT was effective against all tested strains in the agar-well diffusion susceptibility tests

The antibacterial activity of the KT was examined against a broad spectrum of microorganisms in the present study. The results (Table 5) showed that, activity against several gram positive (*S. aureus*, *B. subtilis*, *S. lutea*) , gram negative (*P. aeruginosa*, *E. coli*, *kebsellia species*) bacteria, dermatophytes (M.canis, T.mentagrophytes, T.rubrum) and Candida species was observed with KT. The KT induced a significant growth inhibition zone (44±3.5 mm) at the concentration (50 mg/ml) against gram positive bacteria and 36±2.2 mm against gram negative bacteria. For dermatophytes the maximal inhibition of fungal growth induced by KT was 32±3.5mm, at the higher dose used (75 mg/ml). The positive control, gentamycin induced a significant growth inhibition zone (60±3.8 mm) against bacteria and itraconazole induced a significant growth inhibition zone (55±3.1mm) against dermatophytes and candida species. By the broth microdilution method, it was seen that MICs ranged from 19.5 to 312 μg/ml and MBCs/ MFCs ranged from 39 to 625μg/ml (Table 6). This suggests that the KT represents a useful source of mixtures of antibacterial compounds that may exhibit potential for use as medicine.

The oral and dermal LD_{50} of KT in rats was found to be above 2000 mg of oil/kg of body weight. After a single oral administration of KT at a dose of 2000 mg/kg of body weight to female rats, all animals survived and no toxic symptoms were evident. Single dermal application of KT at a dose of 2000 mg/kg of body weight revealed no toxic effects of the test substance. All animals survived until the scheduled termination of the study. Body weights and body weight gain were inconspicuous during the whole study in all rats, and all animals were normal at the terminal necropsy. In the acute dermal irritation/corrosion study with rabbits, no general toxic effects of KT were observed and

all exposed skin sites were found to be normal at each examination term.

Discussion

Ayurveda is one of the most ancient traditional medicine systems, originated in India. It is still an unexplored treasure. Scientific techniques to develop qualitative and quantitative standards for herbal preparations will enable us to ensure quality, safety and efficacy (Anonymous, 1978; Sharma *et al.*, 1985; Mukherjee, 2002). Present study is an attempt for the same.

Herbal drugs, singularly and in combination, contain a myriad of compounds in complex matrices in which no single active constituent is responsible for the overall efficacy. Initially only a few markers of pharmacologically active constituents were employed to assess the quality and authenticity of complex herbal medicines. However, the therapeutic effects of herbal medicines are based on the complex interaction of numerous ingredients in combination. Thus World Health Organization (WHO), Chinese State Food and Drug Administration (SDA) and the United State Food and Drug Administration (USFDA) does not definitely emphasize the development of chemical assay of all chemical components from herbal medicines and thus, the fingerprinting approach has been recently recognized and accepted for quality assessment of traditional medicines (EMEA, 2007).

Fingerprint of a herbo-mineral medicine is, in practice, a pattern of response produced by pharmacologically active and or chemically characteristic constituents present in the extract on application of an analytical technique. The determination of common peaks/regions in a set of fingerprints could provide useful qualitative and quantitative information on the characteristic components of herbal medicines investigated. The chemical fingerprints obtained by chromatographic and electrophoretic techniques, especially by hyphenated chromatographies, are strongly recommended for the purpose of quality control. Since they might represent appropriately the "chemical integrities" of herbal medicines and therefore be used for authentication and identification of the herbal products and thus may serves as a promising quality assurance tool for herbal medicines. Fingerprint analysis of KT using GS_MS, FTIR and HPTLC represents a comprehensive qualitative approach for the purpose of evaluation of quality and ensuring the consistency of products.

X-Ray powder diffractometry [XRPD] finds widespread application for the identification and quantification of crystalline solid phases. The diffraction pattern of every crystalline form of a mineral is unique in which the *d*-spacing between and the relative intensities of the diffracted maxima can be used for qualitative and quantitative analysis.2θ values of sample and reference should be reproducible by ±0.10°. Each phase produces its XRPD pattern independently of the others. Thus, simultaneous identification of multiple mineral ingredients in a formulation is possible. In recent years, regulatory agencies such as USFDA (USA), Therapeutic Goods Administration (TGA, Australia), Health Canada (Canada) and European Medicinal Evaluation Agency (EMEA, Europe) have realized the potential utility of XRPD as a fingerprinting tool for characterization of mineral components in traditional medicines (EMEA, 2007; Kyono *et al*., 2005). XRPD analysis of KT indicates that crystalline characteristics of the components were not altered significantly and the presence or absence of the component can be evaluated based on characteristic peak.

KT can be an excellent candidate for the treatment of many infectious diseases, including mycosis, due to the increasing development of antimicrobial resistance as well as the appearance of undesirable effects of some antifungal agents (Sharma 1985; Anonymous 2000). Early reports on *Allium sativum* bulb (family, Alliaceae), *Plumbago zylenicum* root (family, Plumbaginaceae), *Alstonia scholaris (*family, Apocynaceae), *Psoralea Corylifolia(*family, Fabaceae) revealed their antimicrobial action. There are reports on the

highest and broadest activity against bacteria and fungi, including yeasts, dermatophytes and non-dermatophytic fungi (Arulmozhi *et al*. 2007; Schmidtke *et al.* (2006); Benkeblia, (2004); Ohta *et al*., 1999; Prasad *et al*., 2004; Ahmad *et al*. (1998)).High psoralen and plumbagin content in the *taila* was reported by Dubey *et al*. (2009).The present study showed that the KT was quite effective against bacteria and, the most common species of dermatophytes that cause superficial fungal infection.

The antimicrobial activity of KT was also evident in the agar-well diffusion and broth microdilution studies. As there was a good correlation between the MICs, MFCs and the agar-well diffusion values of the formulation, it may be concluded that the antimicrobial activity of *taila* preparation could be preliminarily investigated by the agar-well diffusion test for rapid screening (Fontenelle *et al*. 2007).

KT showed a low acute oral and dermal toxicity with an LD_{50} > 2000 mg/kg of body weight (a concentration high above the therapeutic dose) and was not irritating to the skin. Further toxicity studies including acute eye toxicity, skin sensitization, mutagenicity and chronic exposure are needed to determine the complete toxicity profile of KT (Craiga 2004; Buxbaum 2006; Burdock and Carabin 2008).

Owing to its broad spectrum of antimicrobial effect, in-vitro, and low toxicity, the KT proved to be a promising source as new antimicrobial drugs. However, specific pharmacological approaches will be needed in future clinical trials to validate its use as a phytotherapeutic product (Burdock and Carabin 2008, Cross et al, 2008). The preliminary results of the present study demonstrated its broad antimicrobial properties, which make KT a promising tool for topical application in the prophylaxis and treatment of bacterial and fungal infections.

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Table 1. GC-MS Fingerprint of *Kustharaksasa Taila*

No.	Time (min)	Area/Height (sec)	No.	Time (min)	Area/Height (sec)
1.	14.70	2.95	**9.**	25.80	4.33
2.	18.35	6.40	**10.**	28.16	19.50
3.	18.85	7.37	**11.**	29.15	28.62
4.	19.87	8.07	**12.**	30.35	7.01
5.	21.26	7.12	**13.**	32.95	7.81
6.	22.59	4.71	**14.**	35.98	10.74
7.	23.15	6.43	**15.**	37.29	12.15
8.	24.15	3.97			

Table 2. HPTLC Chromatographic Fingerprint of *Kustharaksasa Taila*

No.	Band	Max R_f	Peak area*
1	1	0.02	3118.8±26.31
2	2	0.12	1664.2±12.73
3	3	0.19	2415.8±10.47
4	4	0.23	2074.5±14.92
5	5	0.32	1940.7±12.03
6	6	0.35	3130.2±18.38
7	7	0.44	1907.9±11.37
8	8	0.49	848±6.2
9	9	0.52	2442±19.2
10	10	0.57	2077.6±16.39
11	11	0.65	3231.5±22.1
12	12	0.73	25235.7±63.82
13	13	0.96	9473±96
14	14	1.01	105.5±0.4

* mean value ± standard deviation (n=3)

Table 3. FTIR Fingerprint of *Kustharaksasa taila*

No.	Fatty oils#	mustard oil#	KT#	Interpretation
1.	3025	3069	3064	=CH trans stretching
2.	3006	3007	3007	=CH cis stretching
3.	2953	2949	2958	$CH(CH_3)$ asymmetric stretching
4.	2924	2927	2923	$CH(-CH_2-)$ asymmetric stretching
5.	2854	2856	2851	$CH(-CH_2-)$ asymmetric stretching
6.	1746	1748	1743	-C=O ester Fermi resonance
7.	1653	1654	1650	-C=C- cis stretching
8.	1465	1467	1459	$-CH(-CH_2-, CH_3)$ bending
9.	1377	1378	1374	$-CH(CH_3)$ symmetric bending
10.	1238	1241	1241	$-C-O, -CH_2-$ stretching bending
11.	1161	1165	1156	$-C-O, -CH_2-$ stretching bending
12.	1118	1120	1116	-C-O, stretching
13.	1097	1098	1098	-C-O, stretching

Characteristic FTIR peaks in cm^{-1}.

Table 4 . Characteristic XRPD Fingerprint of *Kalka* in *Kustharaksasa Taila*

No.	% Peak Intensity	Significance	*d*-spacing (Å)	Angle (2θ)	Interpretation
1.	100	1.74	4.78	18.51	Orpiment
2.	74.67	11.48	5.38	16.45	Realgar
3.	84.2	2.38	3.35	26.52	Kajjali
4.	84.29	2.38	3.35	26.52	Sindur
5.	39.45	2.22	3.21	27.72	Kajjali
6.	48.28	1.67	2.86	31.2	Sindur
7.	32.24	1.42	2.81	31.53	Copper sulphide
8.	37	3.32	2.78	32.11	Orpiment
9.	37	3.32	2.78	32.11	Orpiment
10.	16.88	2.33	2.45	36.52	Orpiment
11.	15.77	1.09	2.12	42.46	Kajjali
12.	20.87	0.8	1.98	45.76	Sindur
13.	62.9	1.15	1.9	47.57	Copper sulphide
14.	62.9	1.15	1.83	49.57	Realgar
15.	26.77	1.35	1.68	54.43	Sindur
16.	11.88	1.59	1.3	72.5	Sindur
17.	10.63	1.44	1.1	88.44	Orpiment

Table 5. Antimicrobial susceptibility testing of *KT* by agar well diffusion assay

Strains	**Growth inhibition zones (mm)** ±S.D.(n=3)					
	***KT* (mg/ml)**				**GTM[#¥] (mg/ml)**	**ITZ[#£] (mg/ml)**
	25	**50**	**75**	**100**	**1**	**0.5**
Gram Positive Bacteria						
Staphylococcus epidermidis	21±2.4	44±3.5	TI*	TI*	58±4.2	-
Staphylococcus aureus	25±1.7	48±3.1	TI*	TI*	55±3.1	-
Bacillus subtilis	31±2.9	52±2.5	TI*	TI*	60±3.8	-
Gram Negative Bacteria						
Escherichia coli	23±2.5	42±4.3	TI*	TI*	45±3.7	-
Pseudomonas aeruginosa	19±1.8	36±2.2	TI*	TI*	30±2.9	-
Kebsiellia spp (clinical isolate)	29±2.6	49±3.7	TI*	TI*	42±2.5	-
Dermatophytes						
Candida albicans	8±1.1	23±3.6	36±3.3	TI*	-	46±2.6
Candida tropicalis	7±1.4	18±2.1	32±3.5	TI*	-	44±2.8
Trycophyton mentagrophytes	10±1.2	26±1.8	46±2.4	TI*	-	52±2.7
Trycophyton rubrum	10±1.4	29±2.4	48±3.6	TI*	-	48±3.2
Microsporum canis	08±1.3	16±2.1	33±3.1	TI*	-	55±3.1
Epidermis flocossum	12±1.2	35±2.4	52±4.2	TI*	-	54±3.5

* Total inhibition of growth ; [¥] Gentamycin; [£] Itraconazole

Table 6. *In-Vitro* Antimicrobial Activity of *KT* Determined by Broth Microdilution Method

Strains	*Kustharaksasa Taila* (μg/ml)		Gentamycin (μg/ml)		Itraconazole (μg/ml)	
	MIC 80%	MBC/ MFC	MIC 80%	MBC/ MFC	MIC 80%	MBC/ MFC
Gram Positive Bacteria						
Staphylococcus epidermidis	19.5	39	31.2	62.5	-	-
Staphylococcus aureus	19.5	39	31.2	62.5	-	-
Bacillus subtilis	19.5	39	15.6	31.2	-	-
Gram Negative Bacteria						
Escherichia coli	78	156	31.2	62.5	-	-
Pseudomonas aeruginosa	156	312	125	250	-	-
Kebsiellia spp (clinical isolate)	39	78	31.2	62.5	-	-
Dermatophytes						
Candida albicans	156	312	-	-	1	2
Candida tropicalis	312	625	-	-	2	4
Trycophyton mentagrophytes	78	156	-	-	0.125	0.250
Trycophyton rubrum			-	-	0.125	0.250
Microsporum canis			-	-	0.06	0.125
Epidermis flocossum			-	-	0.125	0.250

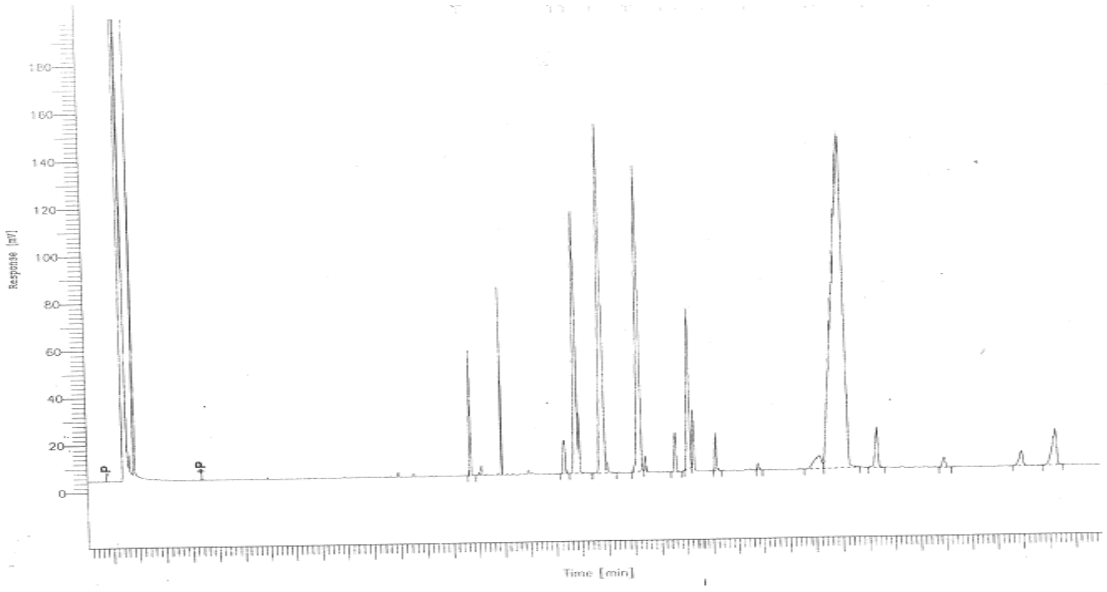

Figure 1. GC-MS Fingerprint of Kustharaksasa Taila

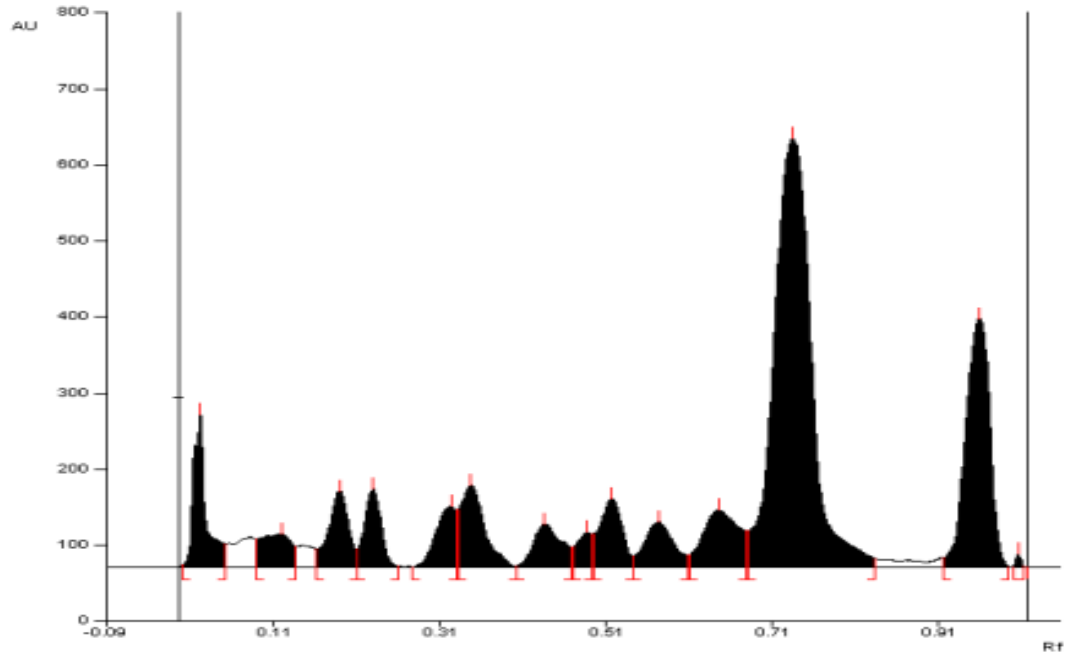

Figure 2. HPTLC Chromatographic Fingerprint of *kustharaksasa taila*

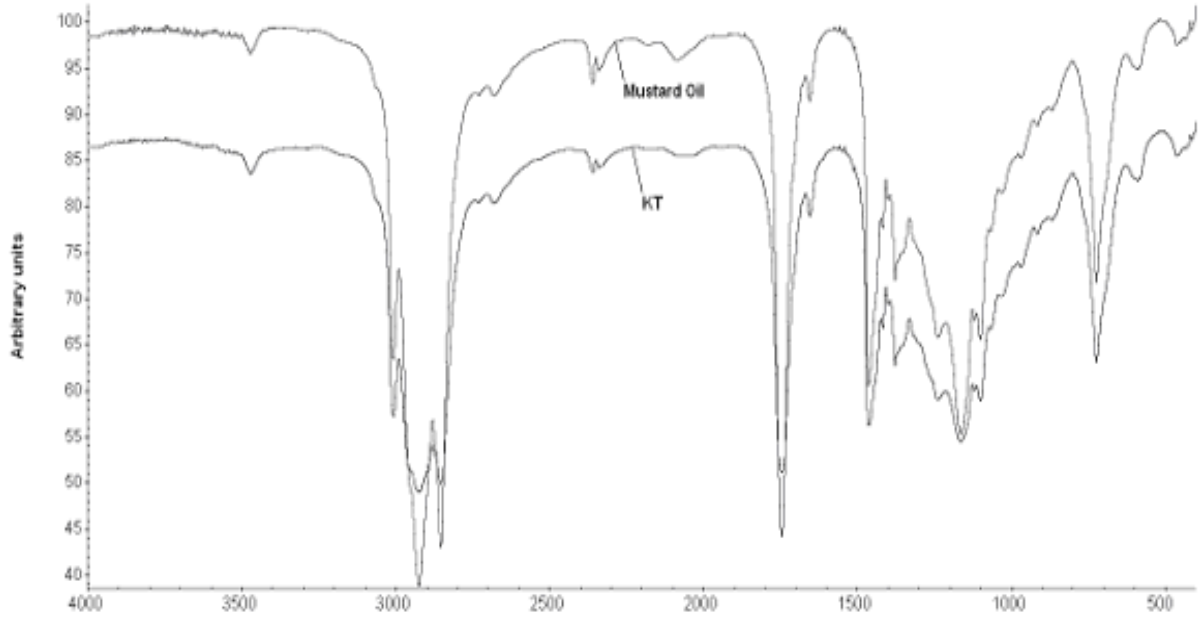

Figure 3 FTIR Fingerprint of Kustharaksasa Taila

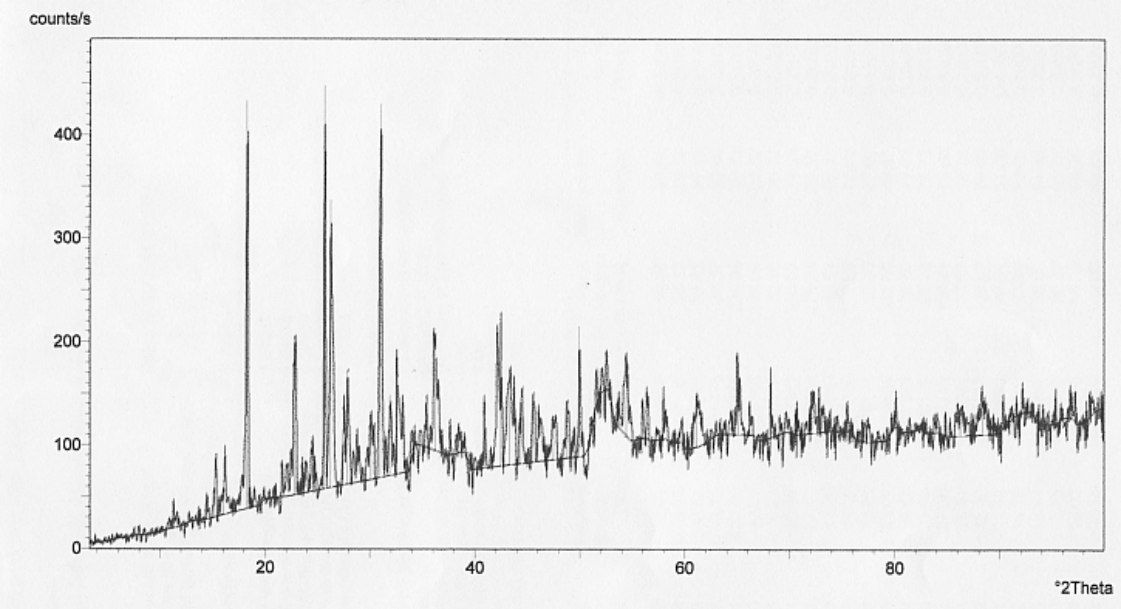

Figure 4.XRPD Fingerprint of *Kalka* in *Kustharaksasa Taila*