Antibacterial and Pharmaco-poeial Studies of Itrifal Usthukhuddus Formulation

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Abstract

Unani formulation Itrifal Usthukhuddus was prepared in laboratory scale as per the guidelines of NFUM (part I) in three batches and was tested against the gram negative eight isolates of *Klebsiella pneumoniae.* The efficacy of drug over the test organisms was studied using the cup plate method. The study revealed the inhibition capacity of the prepared drug on all tested organism irrespective of the strain variation. The activity of the drug was expressed in terms of zone of inhibition and exhibited the MIC value as 6.25mg/ml for most of the organism. The antibacterial study is also supported with the developed pharmacopoeial data and quality control parameters like microbial content, heavy metals, aflatoxin and pesticidial residues laid down by the WHO in usage of traditional medicine.

Keywords: Antibacterial activity, Pharmacopoeial parameters, Quality control parameters.

Introduction

Antimicrobial agents are the miracle drugs in the treatment of infectious diseases. Antimicrobial resistance is the ability of certain microorganisms to withstand the attack by antimicrobials. Development of this uncontrolled rise in resistant microorganism threatens the lives and also urges many pharmaceuticals companies, global health institutions in search of newer potential drugs without implementing side effects and relies on plant based medicines.

In natural products the raw materials like plants, animals and minerals are the vital source in discovery of many novel valuable potential drugs. Presently classical Unani medicines impart a great revolution among the public in treating various diseases. Though the scenario falls like this, it becomes necessary to establish scientific validation in usage of such folklore medicine to achieve the global acceptance. In Unani system of medicine Itrifal Usthukhuddus is a semisolid formulation being prescribed by the Unani physicians for the treatment of various ailments like Muzmin (Sinusitis), Sara (Epilepsy) and Laqwa (Facial Paralysis).

Klebsiella pneumoniae is a gram negative, non motile, encapsulated, lactose fermenting facultative anaerobic, rod shaped bacterium found as a normal flora in mouth, skin and intestine. In general *Klebsiella* infections are seen in patients with weak immune system. The clinical manifestations falls

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like pneumonia, thrombophlebitis, urinary tract infection (UTI), cholecystitis, diarrhea, upper respiratory tract infection, wound infection, osteomyelitis, meningitis, bacteremia and septicemia. New multidrug resistance strains of *Klebsiella pneumoniae* are increasingly emerging and posing a major global health care issue (Vinod Kumar *et al.,* 2011).

Keeping this in view the present study was planned to explore the therapeutic efficacy of the prepared drug Itrifal Usthukhuddus against various strains of *Klebsiella pneumoniae,* to evaluate the pharmacopoeial standards of drug on WHO parameters.

Materials and Methods

Collection and preparation of drug

The drug Itrifal Usthukhuddus used in the study was prepared at the Drug Standardisation Research Unit (DSRU), Chennai. The authenticated good quality raw drugs procured from the local market, Chennai were used in the preparation. The drug was prepared in three batches using the raw drugs namely Halela Siyah, Post-e-Halela Zard, Post-e-Halela Kabuli (*Terminalia chebula* Retz.), Post-e-Balela (*Terminalia bellirica* Roxb.), Aamla (*Emblica officinalis* Gaertn.), Gul-e-Surkh (*Rosa damascena* Mill.), Usthukhuddus (*Lavandula stoechas* Linn.), Bisfayej (*Polypodium vulgare* Linn.), Aftimoon (*Cuscuta reflexa* Linn.), Kishmish (*Vitis vinifera* Linn.) and Raughan-e-Badam (*Prunus amygdalus* Batsch var. Dulcis.), using the guidelines of National formulary of Unani Medicine Part I (Anonymous, 2006).

Collection of microorganism

The microorganisms used in the study were all clinical strains of *Klebsiella pneumoniae,* collected from the diagnostic centers and hospitals of Chennai. The collected isolates of *Klebsiella pneumoniae* were subjected to biochemical reactions in the Department of Microbiology, RRIUM Chennai for further confirmation of the organism (Mackie & McCartney, 1996).

Physico-chemical analysis

Physico-chemical parameters like total ash, acid insoluble ash, solubility in alcohol and water, loss on drying at 105º were performed as per the developed protocol. The bulk density, sugar estimation and *pH* values for 1% and 10% aqueous solution were also carried out (Flow Chart - 1) (Anonymous, 1987).

Thin layer chromatography

Chloroform and alcohol extracts of the drug were developed on precoated silica gel 60 F254 TLC plates (E.Merck) using solvent systems, toluene : ethyl acetate 9:1 and 1: 1 respectively and observed the colour spots at UV-254, UV-366 nm and vanillin-sulphuric acid spraying reagent (Wagner *et al.,* 1984).

Quality control parameters

The microbial load and heavy metal were carried out as per the WHO guidelines (Anonymous, 1998). Aflatoxin and pesticide residues were carried out by standard methods (Anonymous, 2000).

Antibacterial activity

In-vitro antimicrobial susceptibility test was performed using the cup plate method (Anonymous, 1996). Required numbers of Muller Hinton agar plates were prepared and swabbed with eight isolates of the *Klebsiella pneumoniae* and were allowed to stand for few minutes and wells of 6mm diameter were made using the agar gel borer. The wells were loaded with 50µl of the drug at the concentration of 100mg/ml. Dimethylsulphoxide (DMSO) (Howard C.Ansel *et al.* 1969) was used as the solvent. The disc Ampicillin (10mcg/disc) was used as standard for comparison (Anonymous, 1982) and the plates were kept at 37 °C for 18-24 hours. Zone of inhibition was measured using the calipers.

Minimum inhibitory concentration (MIC)

MIC is the lowest concentration of the drug required to inhibit the microorganism was determined by the agar diffusion method (Anonymous, 198210). Petri dishes containing 20ml of Muller Hinton agar media were prepared and swabbed with eight isolates of *Klebsiella pneumoniae*. Required number wells of 6mm diameter were made using the agar gel borer and the drug was added with increasing concentration (50mg/ml, 25mg/ml, 12.5mg/ml and 6.25mg/ml) and was allowed to solidify. The lowest concentration of the drug (MIC) that completely inhibits the growth was determined after overnight incubation at 37°C.

Results and Discussion

Physico-chemical parameters

The results of Physico-chemical parameters are summarized (Table 1).

Thin layer chromatography

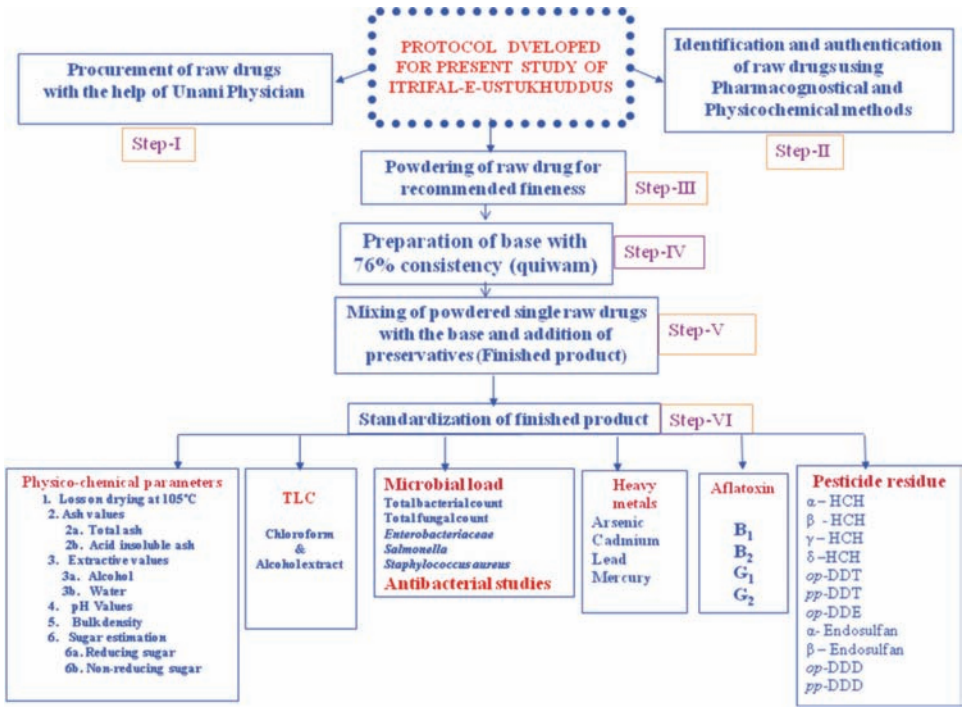
Chloroform and alcohol extract of all the three batch samples shows identical spots in various detector ranges. The R_f values of the chloroform and alcohol extracts are shown (Table 2, 3 & Fig.1, 2).

Quality control parameters

Heavy metals such as lead, mercury, cadmium and arsenic are found below the permissible limit (Table 4). The microbial load also is present within the permissible limit (Table 5). Studies of other parameters like estimation of aflatoxins such as B_1, B_2, G_1 and G_2 and pesticide residue such as organo chlorine group, organo phosphorus group, alachlor, aldrin, chlordane, DDT, endosulfan, heptachlor, lindane and malathion are not detected from the drug (Table 6 & Table 7) .

Antimicrobial activity and MIC

The drug exhibited good antimicrobial activity against all the tested clinical strains of *Klebsiella pneumoniae* at the drug concentration of 100mg/ml with slight variation in zone diameter. The MIC value of the drug also differs and ranged between 6.25mg/ml to 3.125mg/ml against the eight tested strains of *Klebsiella pneumoniae* (Table 8, Chart 1 & Fig 3).



Flow Chart: 1 Protocol for Study

Table 1: Physico-chemical parameters

Parameters Analyzed	Batch Number (n = 3)		
	I	II	III
Extractives			
Alcohol soluble matter	56.81%	57.13%	56.86%
Water soluble matter	67.25%	67.64%	68.04%
Ash			
Total ash	0.87%	0.77%	0.87%
Acid insoluble ash	0.06%	0.11%	0.05%
pH values			
1% Aqueous solution	5.87%	5.73%	5.66%
10% Aqueous solution	4.32%	4.56%	4.46%
Sugar estimation			
Reducing sugar	37.68%	37.92%	37.81%
Non-reducing sugar	12.93%	13.04%	12.98%
Moisture	12.29%	13.17%	12.42%
Bulk Density	1.5342%	1.5496%	1.538%

Table 2: R_f Values of chloroform extract

Solvent system Toluene: Ethyl acetate (9 : 1)	Rf Values		
	UV 254nm	**UV 366nm**	**V. S. Reagent**
	0.93 Light pink 0.74 Light pink 0.66 Pink 0.41 Pink 0.28 Light pink 0.21 Pink 0.14 Pink	0.76 Light blue 0.41 fluorescent blue 0.28Violet	0.92 Blue 0.85 Pink 0.73 Grey 0.65 Dark green 0.58 Dark green 0.40 Dark green 0.28 Grey 0.21 Grey 0.12 Grey

Fig. 1. V. S. Reagent

Table 3: R_f Values of alcohol extract

Solvent system Toluene: Ethyl acetate (1 : 1)	Rf Values		
	UV 254nm	**UV 366nm**	**V. S. Reagent**
	0.94 Light pink 0.66 Light pink 0.41Pink 0.34Pink 0.14 yellowish green 0.10 Pink	0.89 Blue 0.66 Light Blue 0.20 Orange 0.10 Violet	0.86 Grey 0.76 Violet 0.56 Violet 0.46 Grey 0.41 Grey 0.37 Blue 0.20 Grey

Fig. 2. V. S. Reagent

Table 4: Estimation of Heavy Metals

Parameters	Results	WHO/API Limits
Lead	0.1087ppm	10ppm
Cadmium	Nil	0.3ppm
Mercury	0.0104ppm	1ppm
Arsenic	Nil	3ppm

Table 5: Estimation of Microbial load

Parameters	Results	WHO Limits for internal use
Total Bacterial Count (TBC)	$2x10^2$cfu/g	$1x10^5$cfu/g
Total Fungal Count (TFC)	Absent	$1x10^3$cfu/g
Enterobacteriaceae	Absent	$1x10^3$cfu/g
Escherichia coli	Absent	$1x10^1$cfu/g
Salmonella spp	Absent	Absent
Staphylococcus aureus	Absent	Absent

Table 6: Estimation of Aflatoxin

Aflatoxin	Results
B1	Nil
B2	Nil
G1	Nil
G2	Nil

Table 7: Estimation of Pesticidal residue

S. No.	Pesticide residues	Results
1	Organo Chlorine Group	ND
2	Organo Phosphorus Group	ND
3	Acephate	ND
4	Chlordane	ND
5	Dimethoate	ND
6	Endosulphan	ND
7	Endosulfan	ND
8	Endosulfon	ND
9	Ethion	ND
10	Endosufon sulphate	ND

11	Fenthion	ND
12	Heptachlor	ND
13	Lindane	ND
14	Methoxychlor	ND
15	Phorate sulfoxide	ND
16	Phorate sulfone	ND
ND – Not detected		

Table 8: Determination of Minimum Inhibitory Concentration

S. No.	Microorganisms	Zone diameter in mm (n=2)						Std (amp) 10mcg/ml
		100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	3.125 mg/ml	
1	*Klebsiella pneumoniae* KP-I	17	15	10	9	-	-	S (15mm)
2	*Klebsiella pneumoniae* KP-II	16	15	14	12	11	-	S (22mm)
3	*Klebsiella pneumoniae* KP-III	15	13	12	11	9	-	S (17mm)
4	*Klebsiella pneumoniae* KP-IV	16	14	12	10	8	-	S (17mm)
5	*Klebsiella pneumoniae* KP-V	18	15	11	9	-	-	S (18mm)
6	*Klebsiella pneumoniae* KP-VI	17	16	14	11	10	-	R
7	*Klebsiella pneumoniae* KP-VII	18	16	13	12	10	-	S (20mm)
8	*Klebsiella pneumoniae* KP-VIII	15	13	12	10	8	-	S (19mm)
S – Sensitive; R- Resistant; Amp - Ampicillin								

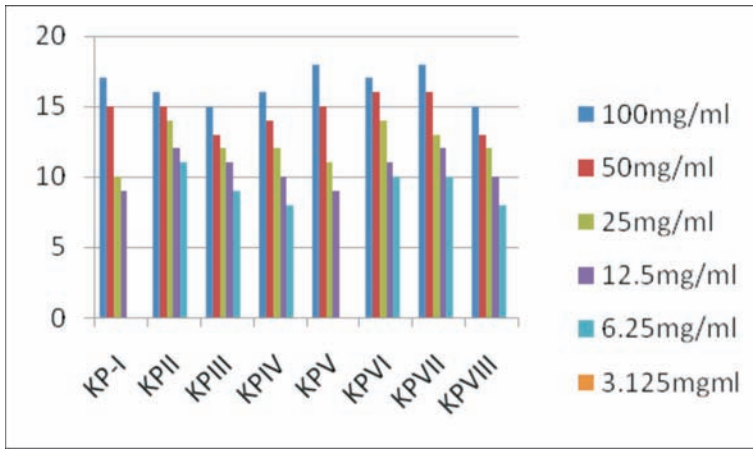
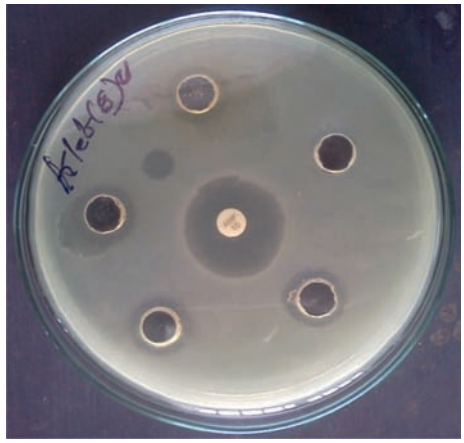
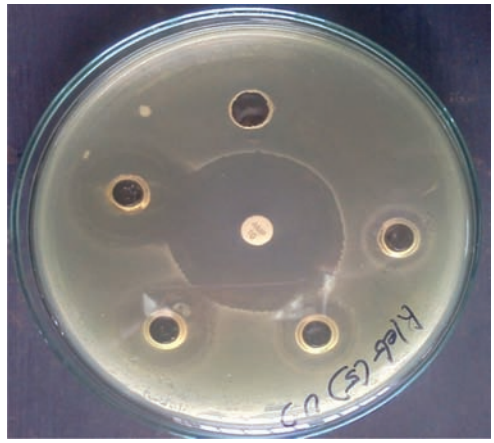


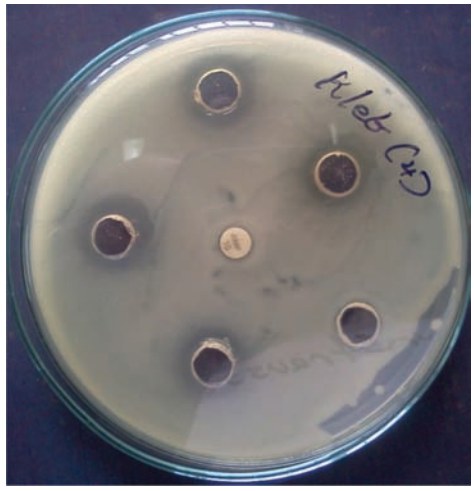
Chart 1. Antibacterial activity



Klebsiella pneumoniae - I



Klebsiella pneumoniae - II



Klebsiella pneumoniae - VI

Drug Conc.	
1.	50mg/ml
2.	25mg/ml
3.	12.5mg/ml
4.	6.25mg/ml
5.	3.125mg/ml
6.	Std (Amp-10µg/ml)

Fig. 3: Plates showing antibacterial activity

Conclusion

Results of present investigations reveal that formulation prepared is free from Microbial growth and Heavy metals, Aflatoxins, Pesticidial residues are within permissible limits. The antibacterial study revealed that Itrifal usthukhuddus is effective herbal formulation to treat infections caused by organism *Klebsiella pneumoniae*.

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