ORIGINAL RESEARCH PAPER

Microbiology

OCCURRENCE OF CANDIDA SPECIES AS AN OPPORTUNISTIC PATHOGEN IN IMMUNOCOMPROMISED HOST AND TO STUDY THE ANTIMICROBIAL POTENTIAL OF HERBAL EXTRACTS TOWARDS THESE ISOLATES.

KEY WORDS: immune compromised, antimicrobial, opportunistic pathogen,

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ABSTRACT

It is observed that 60-90% of the patients have some underlying immune suppressive condition such as chronic steroid use, solid organ transplantation, malignancy or human immunodeficiency virus (HIV) infection, the growing problem of mucosal and systemic candidiasis reflects the enormous increase in the number of such patients at risk and the increased opportunity that exists for Candida species to invade tissues normally resistant to invasion .These patients are in a state in which the immune system's ability to fight infectious disease is compromised or entirely absent and become vulnerable to opportunistic infections, in addition to normal infections that could affect everyone. Widespread usage of antifungal has rapidly led to the increasing cases of drug resistance which emerges as a threat to the antifungal therapy and therefore there is an urgent need for novel therapies against this pathogen. The use of plants as sources of pharmaceutical drugs that are used in traditional medicine need to be tested for its antimicrobial action towards these isolates

INTRODUCTION

Candidiasis is an infection caused by species of the genus Candida, predominantly *Candida albicans.* This disease usually occurs during the first year of life and in patients with immune system dysfunction. It affects both males and females and may be associated with an inherited defect of the cell-mediated immune system that allows auto antibodies to develop against target organs.

Candida species are frequently part of the human body's normal oral flora. Immunosuppressive conditions result due to treatment with antibiotics and lead to eliminating the yeast's natural competitors for resources. In such immune compromised patients, Candida can affect the oesophagus with the potential of becoming systemic, causing a much more serious condition-Candidemia ***Barelle CJ, et. al, 2006***

The growing problem of mucosal and systemic candidiasis reflects the enormous increase in the number of patients at risk and the increased opportunity that exists for Candida species to invade tissues normally resistant to invasion. Serious life-threatening infections are being reported with an ever-increasing array of pathogens, including the well-known opportunists *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* Herbal drugs have made their importance felt in the last few decades whose prevalence is continuously increasing in both developing and developed countries due to their natural origin and lesser side effects. The antimicrobial efficacy attributed to some plants in treating diseases has been beyond belief and needs to be exploited.

The flowers of *Calendula officinalis* contain a large number of phytochemicals that include flavanol glycosides, triterpene oligoglycosides, oleanane-type triterpene glycosides, saponins, and a sesquiterpene glucoside which exert antimicrobial activity.Turmeric contains a variety of bioactive substances called curcuminoids known to a possess antibacterial activity.

Clove buds are known to be rich in eugenol, are used in dental treatments as analgesic also known to have potential antimicrobial activity.

Use of these extracts in treating these opportunistic infections can prove to be useful in patients with low immune response, especially those which have been overdosed with heavy third generation antibiotics for treating other ailments. Secondary plant metabolisms include products that aid in the growth and development and are a wide range of compounds from different metabolite families. These compounds are not only essential for the maintenance of life of the plant but are often involved in plant protection against biotic or abiotic stress ***(Hattenschwiler and Vitousek, 2000).*** These mainly include Terpenes-- derived from the C5 precursor isopentenyl diphosphate (IPP), Alkaloids--derived from amino acids, Phenolics--shikimate pathway or malignant/acetate pathway. In "The Annual Plant Review: Functions and Biotechnology of Plant Secondary Metabolites" it has been shown that, plants have developed effective defence strategies to protect themselves from Phyto-pathogenic microbes in their environment. Disease resistance in plants depends on the activation of coordinated and multi component defence mechanisms. One of the mechanisms is their ability to accumulate low-molecular-weight compounds (secondary metabolites) with high antimicrobial activities, such as alkaloids, coumarins, flavonoids, poly acetylenes, Quinones, tannins and terpenes ***(Hattenschwiler and Vitousek, 2000); Michael and Reichling 2010).***

Calendula officinalis: Flowers of Calendula officinalis contain a large number of phytochemicals that include flavonol glycosides, triterpene oligoglycosides, oleanane-type triterpene glycosides, saponins, and a sesquiterpene glucoside .Moreover, calenduline and oleanolic acid glycosides, sterol glycosides, alpha-and beta-amyrin, taraxasterol, lapel, brain, faradiol, arnidiol, erythrodiol, Calenduladiol, coflodiol have been found in various parts of the plants ***(Khan et al. 2011***).Calendula officinalis was evaluated previously for its antimicrobial activity by ***Iqbal Hussain (2012***).

Syzygium aromaticum: *Eugenol* comprises 72-90% of the essential oil extracted from Cloves and is the compound responsible for aroma. Other important essential oil constituents are acetyl eugenol, beta- caryophyllene, vanillin crategolic acid, tannins (bicornin, gallotannic acid), methyl salicylate, flavonoids eugenin, kaem-pferol, rhamnetin, and eugenitin. It also contains triterpenoids like oleanolic acid, campesterol and several sesquiterpenes. The major components identified by using gas chromatography-mass spectroscopy were eugenol (71.56 %) and eugenol acetate (8.99%). p-Cymene5-Hexene-2-one,3 Thymol, Caryophyllene oxide,Guaiol, Benzene-1-butylheptyl,Hexadecanoic acid , 9,17-Octadeca-dienal , Octadecanoic acid butyl ester ,Phenol-4-(2,3-dihydro-7 -

methoxy-3-methyl-5-(1-propenyl)-2 –benzofurane, and Vitamin E***(Jadhav et al., 2004).*** Cloves consist of a significant amount of proteins, iron, carbohydrates, calcium, phosphorus, potassium, sodium and hydrochloric acid. They are also rich in vitamins like A and C, manganese, and dietary fibre that can help in metabolism. ***(Ahmad et al., 2005; Pawar and Thaker 2009).***

Curcuma longa

Turmeric contains protein (6.3%), fat (5.1%), minerals (3.5%), carbohydrates (69.4%) and moisture (13.1%). The essential oil (5.8%) obtained by steam distillation of rhizomes has a-phellandrene (1%), sabinene (0.6%), cineol (1%), Borneo (0.5%), zingiberene (25%) and sesquiterpenes (53%). Curcumin (diferuloylmethane) (3–4%) is responsible for the yellow colour, and comprises curcumin I (94%), curcumin II (6%) and curcumin III (0.3%). Dimethoxy and bisdemethoxycurcumin derivatives of curcumin have also been isolated. Turmeric contains a variety of bioactive substances called curcuminoids. The most active component is curcumin, an orange-yellow volatile oil that includes three curcuminoid, atlantone, and zingiberone ***(Mahady et al., 2002).*** As crude turmeric extracts possess antioxidant properties, it can act as a scavenger of oxygen free radicals. In vitro, curcumin significantly inhibits the generation of reactive oxygen species (ROS) like superoxide anions, H2O2 and nitrite radical generation by activating macrophages, which play an important role in inflammation.

OBJECTIVES:

i. To study the incidence of this opportunistic pathogen in individuals.
ii. Identification of the etiological agent form samples collected from immune compromised individuals.
iii. To prepare Aqueous and alcoholic extracts of the selected herbs and study in-vitro antibacterial effect of the extracts,
iv. To study the Anti-bio gram pattern of these isolates against conventional antibiotics and Herbal extracts as per the CLSI guidelines.
v. The study aims to provide scientific validity and credence to the ethno medicinal use of plant extracts and highlights the usefulness in controlling these infections.

METHODOLOGY:

i. Sample collection: Oral swabs will be collected from individuals, after noting their brief infection history along with the past antibiotics administered.
ii. Inclusion Criteria: Patients of all age groups and will be considered clinically suspected to have an infection and showing suspicion of infective etiology and are able to produce a brief, relevant history.
iii. Exclusion Criteria: Samples from healthy individuals and not administered with antibiotics will be rejected
iv. Identification: The analytical profile index or API system will be used for quick identification of clinically relevant isolates after a prior confirmation.
v. Herbal extraction and antibacterial testing: Metabolites from the active part of the above-mentioned plants will be extracted and tested against the isolated pathogens by the Standard Disc diffusion technique

- Wet mounts: A wet mount of a small amount of sample in a liquid medium was prepared using either of the two liquids. Normal saline is a physiologic solution, so cell membranes are preserved, and vital activities remain undisturbed or Potassium hydroxide (KOH) dissolves cell membranes and other biologic materials, but not the cellulose found in the cell walls of fungi, everything except the Candida. This property makes it particularly useful in identifying Candida.
- Identification: The analytical profile index or API system was used for identification of clinically relevant isolates after a prior confirmation. Incubating the strips at 370 C for 24 hours and using the appropriate reagents the isolates were identified up to the generic level.
- KB006 Hi-media is a standardized colorimetric identification system utilizing twelve conventional biochemical tests that can be used for identification and differentiation of Candida species. The tests are based on the principle of pH change and substrate utilization. On incubation, organisms undergo metabolic changes which are indicated by a spontaneous colour change in the media. The complete list of organisms that can be identified with this system is given in the identification index provided with the kit.
- Herbal extraction

i) Aqueous extraction: Dried plant material was extracted in distilled water in a final ratio of 1: 16 using reflux condenser, this was followed by concentration of the extract.
ii) Solvent (Methanol) Extraction-The principle of solid, liquid extraction is that when a solid material was exposed to a solvent, the active principle (medicinal ingredient) present in the plant material, if soluble in the solvent, actively moved from solid material into the solvent.
iii) Preparation of the working extract: 100 mg of the extract was weighed using electronic balance and was dissolved in100 ml methanol (for Alcoholic extract), and 100 ml of DMSO or Physiological saline (which ever gave best dissolution was used for the Aqueous extracts). This resulted in a concentration of 1.00 mg/ml of the extract

Spray gas	Sample-solvent type	Dosage speed	Pre dosage volume	Syringe size	Application position Y	Band length
Inert gas	Methanol	150 µl/s	0.2 µl	100 µl	8 .0mm	8 .0mm

- HPTLC fingerprinting profile

Herbal extracts contain many constituents; hence it is important to confirm the bioactive compound. It is critical to obtain reliable chromatographic fingerprint that represent pharmacologically active and chemically characteristic components present in the extract. The HPTLC fingerprinting profile is an important parameter of herbal drug standardization for proper identification of medicinal plants. The extracts along with the standard markers were subjected to HPTLC. Markers served to calculate the quantity of herbal substances or herbal preparations in the herbal medicinal product.(SYSTEM USED FOR HPTLC: Sample application; CAMAG Lino mat) Spray gasSample-solvent typeDosage speedPre dosage volumeSyringe sizeApplication position YBand lengthInert gas Methano l150 µl/s0.2 µl100 l8 .0mm8 .0mm

- Disc diffusion assay of the herbal extracts :The density of the inoculum was adjusted to (1.5x10 8 CFU/ml), inoculated onto the entire surface of a sterile Saburauds agar plate with a sterile cotton-tipped swab to form an even lawn. Sterile paper disc, (6mm diameter) were placed on the surface of each Saburauds agar plate using a sterile pair of forceps and impregnated with 20 µl of the herbal extract. Then the plates were incubated aerobically at 25°C for 72 hrs and the diameter of zone inhibition was measured. The antimicrobial activity results were expressed in term of the diameter of the zone of inhibition and <9mm zone was considered as inactive; 9-12mm as partially active; while13-18mm as active and >18mm as very active (Junior and Zanil, 2000; Pundir and Jain 2011). The mean and standard deviation of the diameter of inhibition zone were calculated

RESULTS

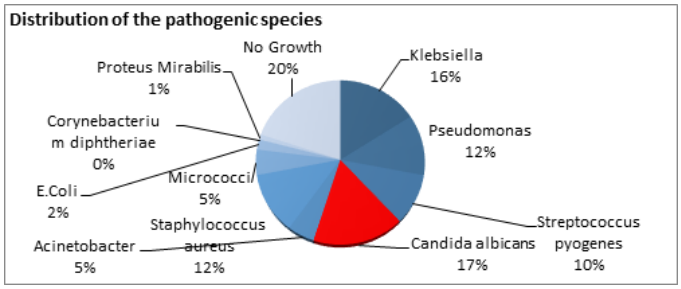


TABLE ;1: Identification of Candida spp using KB006 Hi-media is a standardized colorimetric identification system

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylose	+	+	+	+	+	+	V	+	+	+	+	+	+	+	V	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-
Trehalose	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	-	+
	Ca	Ca	Ca	Ca	Ca	Ct	Ct	Ca	Ca	Ct	Ca	Ca	Ca	Ca	Ct	Ct	Ca

KEY : Ca- C.albicans; Ct-C.tropicalis '+'-Positive result '_'Negative result

TABLE :2: Microscopic study and herbal sensitivity of isolates

SAMPLE		MICROSCOPY	HERBAL SENSITIVITY IN mm					
			AL-AQ	AL-AC	CL-AQ	CL-AC	TU-AQ	TU-AC
C1	C.albicans	EC+ PC+	8	12	6	22	8	10
C 2	C.albicans	PC<10 EC <10 PSEUDO HYPHAE	8	14	6	28	15	12
C 3	C.albicans	Oval yeast cells	8	12	12	22	8	10
C 4	C.albicans	PC10-12/EC>25	10	16	6	28	6	12
C 5	C.albicans	Oval yeast cells	8	12	14	28	8	12
C 6	C.tropicalis	PC++/PSEUDOMY	6	14	15	28	10	12
C 7	C.tropicalis	PC<25 EC>10	10	14	18	30	12	16
C 8	C.albicans	PC<10 EC <10 PSEUDO HYPHAE	8	16	6	25	10	18
C 9	C.albicans	Oval yeast cells	8	16	18	26	8	14
C 10	C.tropicalis	Oval yeast cells	8	18	14	36	10	16
C 11	C.albicans	Oval yeast cells	10	14	12	22	8	12
C 12	C.albicans	Oval yeast cells	8	16	14	22	8	12
C 13	C.albicans	Oval yeast cells	10	16	18	30	6	14
C 14	C.albicans	Oval yeast cells	8	14	6	25	10	16
C 15	C.tropicalis	PC+EC	8	16	14	22	8	12
C 16	C.tropicalis	PC<10 EC <10 PSEUDO HYPHAE	8	16	14	22	8	12
C 17	C.albicans	PC<10 EC <10 PSEUDO HYPHAE	9	16	18	26	8	14
		MEAN (mm)	8.3	14.9	12.4	26	9.4	13.1

HPTLC Fingerprinting Profile

A-calendula :

Table :3 : Application Parameters

Instrument application	CAMAG Linomat 5	Sequence	
Spray gas	Inert gas	Syringe size	100 µl
sample solvent type	methanol	Number of tracks	6
Dosage speed	150 µl /s	Application position	8.0 m.m.
Pre dosage volume	0.2µl	Band length	8.0 m.m.
Application position	8.0 mm		
Solvent front position	80.0 mm		

Table: 4: Detection Parameters- Lamp: D2/ Wavelength: 254 Nm

Tracks	1				2				3	4	5	6	
Applied sample	Alcoholic extract								Std Calendulin		Aqueous extract		
Applied volume	10µl				20µl				10µl	20µl	10µl	20µl	
Rf	0.31	0.44	0.55	0.81	0.32	0.45	0.55	0.60	--	--	0.63	0.63	0.8
Area of the peak	1327.4	1663.6	2732.4	270.1	2818.6	3517.4	5052.0	460.0	--	--	136.2	517.7	103.2

As the standard failed to show bands, the $R_{f\ values}$ could not be determined. The alcoholic extracts showed distinct bands and on referring to standard R_f values, it could indicate the presence of Lutein (xanthophyll a naturally occurring carotenoid), lycopene. Comparing with standard references it can be correlated to the presence of Lutein (Rf 0.817) as in the visible spectrum the extract showed a Maximum absorbance at 427nm Comparable to Standard Lutein-440nm

B-clove:

Table :6: -application Parameters

Instrument application	CAMAG Linomat 5	Sequence	
Spray gas	Inert gas	Syringe size	100 µl
sample solvent type	methanol	Number of tracks	5
Dosage speed	150 µl /s	Application position	8.0 mm
Pre dosage volume	0.2µl	Band length	8.0 mm
Application position	8.0 mm		
Solvent front position	80.0 mm		

Table :7: Detection Parameters- Lamp: D2/ Wavelength: 254 Nm

Tracks	1	2	3	4	5
Applied sample	Aqueous extract		Std Eugenol		
Applied volume	5µl	10µl	5µl	5µl	10µl
Rf	---	--	0.46	0.45	0.44
Area of the peak	--	--	13946.6	1901.4	3292.1

Table :8 : Detection Parameters- Lamp: Hg/ Wavelength: 366 Nm

Tracks	1	2	3		4	5	
Applied sample	Aqueous extract		Std Eugenol		Alcoholic extract		
Applied volume	5µl	10µl	5µl		5µl	10µl	
Rf	--	--	0.11	0.46	--	0.04	0.45
Area of the peak	--	--	967	1710.4	--	201.6	627.9

Presence of Eugenol in the methanolic extracts was confirmed as Rf -0.46 was seen in standard as well as in methanol extract. UV absorption spectra also showed similar peaks of maximum absorption in extract and standard Eugenol

C-Turmeric

Table :9 : Application Parameters

Instrument application	CAMAG Linomat 5	Sequence	
Spray gas	Inert gas	Syringe size	100 µl
sample solvent type	methanol	Number of tracks	4
Dosage speed	150 µl /s	Application position	8.0 mm
Pre dosage volume	0.2µl	Band length	8.0 mm
Application position	8.0 mm		
Solvent front position	80.0 mm		

Table :10: Detection Parameters- Lamp: D2/ Wavelength: 254 Nm

Tracks	1		2			3			4		
Applied sample	Aqueous extract		Alcoholic extract			Alcoholic extract			Std Curcumin		
Applied volume	15µl		5µl			10µl			10µl		
Rf	0.22	0.86	0.23	0.27	0.87	0.23	0.28	0.88	0.24	0.29	0.88
Area of the peak	6573	33.2	8459	2330	19.2	4450	2105.	69.3	2507	2568	93.7

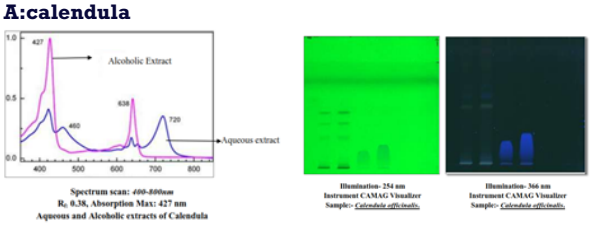
Aqueous extract showed the presence of constituents other than Curcumin, peaks with R_f -0.27 and R_f-0.88 were found in aqueous and alcoholic extracts but the alcoholic extract showed a distinct peak Indicating better extract of the active marker.

Table ;11: Detection Parameters- Lamp: Hg/ Wavelength: 366 Nm

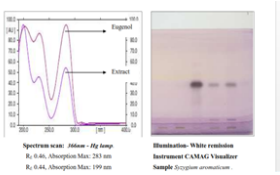
Tracks	1	2			3			4		
Applied sample	Aqueous extract	Alcoholic extract			Alcoholic extract			Std Curcumin		
Applied volume	15µl	5µl			10µl			10µl		
R_f	0..22	0.12	0.23	0.27	0.87	.12	0.23	0.24	0.29	0.88
Area of the peak	4573	367.2	8459	4530	64.2	190	037.62	0508	2653	87.5

Image 1 : Chemical Profiling –

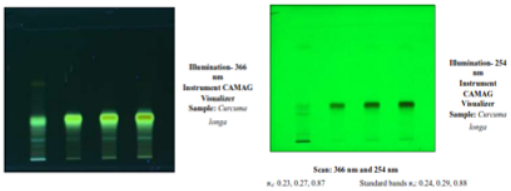
A:calendula



B:CLOVE



C :TURMERIC



DISCUSSION

Increase in fungal pathogens 17 % was observed in the present study. Invasive fungal infections along with limited therapeutic options and emergence of MDR together build a burden on patients with compromised immunity. Due to increasing prevalence in various patient groups, Candida spp. has gained remarkable importance and among all Candida spp., *Candida albicans* was most frequently isolated from blood and tissue samples of affected patients who had administered prolonged antibiotic treatment. Search for novel therapeutic alternatives has emerged due to clinical needs for novel antifungal agents with broad spectrum activity and minimal toxic effects on the host. Plant products have been used since ages in traditional medicines. Due to their safe human use, in recent times there has been lot of efforts for developing therapeutic options using molecules from plant sources.

EFFICACY OF THE PLANT EXTRACTS TESTED

The objective of this study was to evaluate the *in-vitro* anti-fungal activity of crude aqueous and methanolic extracts of Calendula flowers, Clove buds and Turmeric rhizomes specifically towards the Candida isolates

Calendula: The extraction was done using water and methanol. It was observed methanol enabled more efficient extraction of flavonoids, which in accordance to scientific literatures responsible for the active potential qualities in its

curative action. This result was consistent with the result previously cited by ***Monica et al., (2012).*** Phytochemicals in particular phenolic acids and flavonoids, compounds extracted from *Calendula officinalis* are responsible through their antioxidant activity ***(Preethi et al., 2009).*** In the present study Calendula has exhibited potent antibacterial action on Candida species Statistical analyses have confirmed the efficacy of Calendula in restricting candida growth. Calendula is one of the most valuable medicinal plants because a variety of Phytochemicals such as, terpenoids, flavonoids (particularly patulitrin and patuletin), coumarins, Quinones, carotenoids (lutein) and other compounds are present in this plant. The mechanisms underlying these possible effects are poorly understood.

Clove: The active constituents of Clove are known to possess antibacterial activities against anaerobic periodontal oral pathogens, ***(Abe et al., 2003).*** It has been hypothesized that the inhibition involves phenol compounds. These compounds sensitize the phospholipid bilayer of the microbial cytoplasmic membrane and leads to loss of ions and reduction of membrane potential. It is also proposed that there is collapse of the proton pump and depletion of the ATP pool in the cell. Also, the increased permeability leads to leakage of cellular proteins and lipids. ***(Minami et al., 2003;).*** The mechanism of this phenolic toxicity towards fungi is based on the inhibition of fungal enzymes, which contain –SH groups in their active sites. Inhibition of *Candida* isolates in our study can be correlated with results from ***Park (2007), Chaiebk et al., (2007),***

Turmeric : Research on the chemistry of *Curcuma longa,* Turmeric contains phenolic compounds called *Curcuminoids*, typically three major curcuminoids: curcumin (curcumin I), demethoxycurcumin (curcumin II) and bis-demethoxycurcumin (curcumin III), possesses all the bio-protective properties of turmeric ***(Thomas et al., 2014).***The results of the present study are in accordance with results by ***Song E.K (2001)*** where Curcumin fraction was successful in suppressing growth of Candida species as successful inhibition of most of the clinical isolates of *Candida* species was seen. ***(AraúJo et al., CC,2001;Van Wyk et al.,2004).*** *Along with the antibacterial activity, antioxidant activity, anti-inflammatory activity is comparable to steroidal and non-steroidal drugs. Thus, use of turmeric can be of additional use to improve the health status of an individual in a respiratory infection*

SUMMARY

- Calendula was effective in the methanol extraction, especially in restricting *Candida* growth as compared to the aqueous extract. Though presence of calendulin could not be confirmed, flavonoids (particularly patulitrin and patuletin) or lutein could be responsible for effective bacterial inhibition in the present study.
- Clove proved to be the most effective antibacterial agent. This antibacterial efficacy was attributed to the Eugenol in the extracts, the fact that was confirmed by performing HPTLC
- Turmeric extract with a distinct content of Curcumins showed lower bio activity. Turmeric exhibited a lower inhibition of nonpathogenic isolates in comparison to pathogenic isolates It can thereby be useful to restore the critical balance of normal flora when used in treating upper respiratory infections

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