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QUALITY EVALUATION OF A PHYTOPHARMACEUTICAL BY HPTLC AND SCREENING OF ITS IMMUNOSTIMULANT & ANTIMICROBIAL ACTIVITIES

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ABSTRACT

The present work includes the HPTLC quality evaluation of a phytopharmaceutical (immunostimulant & expectorant) formulation prepared from three herbs namely *Ocimum sanctum* (tulsi), *Allium cepa* (red onion) *and Mentha piperita* (mentha) as per the guidelines of Indian Pharmacopoeia Commission, Ministry of Health and family welfare, Govt. of India. The latest direction of Indian Pharmacopoeia commission indicates for the inclusion of a minimum of four therapeutic or analytical markers in a phytopharmaceutical formulation (PPF). In the present work eugenol, menthol and quercetin were used as the markers for the herbal drugs *Ocimum sanctum, Allium cepa and Mentha piperita* respectively.

The Herbovigillance part of the work can give information specific to herb-drug interactions for the patients taking these kinds of preparations along with conventional drugs in the post covid-19 pandemic period. Herbovigillance protocols were framed for the patients under anti-diabetic drugs, anti-coagulants, anti-allergic drugs, and anti-hypertensives for monitoring carefully if taking the prepared phytopharmaceutical formulation along with these conventional drugs.

The antimicrobial activity studies showed zone of inhibition of 11.8 mm and 10.6mm for *E. coli* (gram-ve) and *Staph.aures* (gram +ve) respectively by PPF. The percentage immunostimulation was found to be 64% and 82% for the PPF at the dose of 50mg/ml and100 mg/ml respectively.

The HPTLC analysis of the phytopharmaceutical formulation indicated that, an amount of 0.008 mcg of eugenol (Rf value: 0.83), 0.008 mcg of quercetin (Rf value: 0.73), 0.004 mcg of menthol (Rf value: 0.59) are present per mcg of *Ocimum sanctum, Allium cepa* and *Mentha piperita* extracts respectively. The results can give an insight for herbal manufacturers for the quality development of phytopharmaceutical formulations.

Keywords: Phytopharmaceutical formulation, HPTLC, *Ocimum sanctum, Allium cepa, Mentha piperita,* Immunostimulant, Antimicrobial activity, Herbovigilance.

1.INTRODUCTION

Phytopharmaceutical compounds are normally derived from the purified and standardized extract obtained from the crud e drug. Phytopharmaceutical formulation (PPF) are the herbal formulations which are categorized as the pharmacopeial reference standards thus it differs from AYUSH medicine. As per the guidelines of Indian Pharmacopoeia Commission, Ministry of Health, Government of India[1,2], a phytopharmaceutical formulation can have a maximum of four herbs/or its extracts along with their specific therapeutic or analytical markers for quality evaluation. Normally PPF standardization has done by adding standardized herbal extract from the selected herbals along with defined preservatives[3, 4].

The crude drugs were selected from a licensed local vendor and authenticated by its Pharmacognostical parameters[5,6]. Herbal drugs namely *Ocimum sanctum* (tulsi), *Mentha piperita* (mentha), and *Allium cepa* (red onion) were selected for their HPTLC quality evaluation[7] in the present study. Herbovigilance protocols were prepared with respect to the individual herbals added in the PPF.

2. MATERIALS AND METHODS

Prior to the manufacture of herbal formulations, crude drugs selected must be size-reduced in order to facilitate the complete extraction. All the plants were procured from the local market and authenticated at the Department of Pharmacognosy and Phytochemistry, PPG College of Pharmacy, Coimbatore. All the collected herbals were shade dried and powdered. In order to authenticate the crude drug powder, the tissues of diagnostic importance and phytochemical analysis were carried out.

2.1. Tissues of diagnostic importance in the crude drug powder[27]

The powder microscopy of *Ocimum basilicum* showed presence of numerous glandular simple trichomes of average length 101 μm, calcium oxalate crystals, oil globules, Diacytic stomata, multicellular trichomes and fibers. The powder microscopy of *Mentha piperita* showed the presence of cluster crystals of calcium oxalate, starch grains and oil globules scattered as such throughout or embedded in the parenchymatous cells; fragments of longitudinally cut annular and pitted vessels. The major tissue of diagnostic importance is the multi cellular covering trichomes and glandular trichomes. The powder microscopy of *Allium cepa* showed the presence of parallel, longitudinal cells with nucleus inside.

2.2. Phytochemical examination of the extracts[26]

The dried and powdered plant material (100g) was Soxhlet extracted with ethanol and the percentage yield obtained from each crude drug was calculated after recovering the solvent completely by rotary vacuum evaporator. The crude drug extracts were examined by qualitative chemical tests using specific chemical reagents. The results are reported in Table 2, Table 6.

Table 1: Herbs selected for the phytopharmaceutical preparation[27, 28]

Herb	Botanical Name	Major Phytoconstituents	Types of Phytoconstituents	Uses
Tulsi	*Ocinum sanctum*	Eugenol, Methyl eugenol	Terpenoids	Anti-bacterial Immunostimulant, Antioxidant
Mint	*Mentha piperita*	Menthol	Terpenoids	Anti-bacterial, Cooling agent
Red onion	*Allium cepa*	Quercetin	Flavonoid	Anti-bacterial, bronchodilator

Table 6: Phytochemical screening studies of the extracts[26,29]

Phytoconstituents tested	**TLE**	**MLE**	**ACE**
Fixed oil/fat	-	-	-
Proteins	-	-	-
Carbohydrates	-	-	-
Tannins	-	-	-
Flavonoids	-	-	+
Saponins	-	-	-
Alkaloids	-	-	-
Anthracene glycosides	-	-	-
Cardiac glycosides	-	-	-
Cyanogenetic glycosides	-	-	-
Alkaloids	-	-	-
Terpenoids	+	+	-
Steroids	-	-	-

MLE: Mentha leaf extract, TLE: Tulsi leaf extract, ACE: *Allium cepa* extract

2.3. Phytopharmaceutical preparation

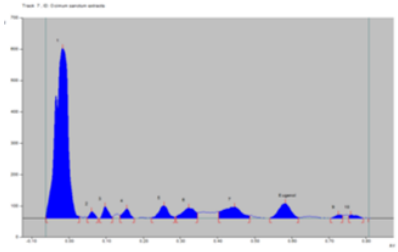
Each crude drug (100g) was extracted with ethanol in Soxhlet apparatus and collected the respective extract and dried. The percentage yield of each extract was calculated. For 100 ml of the cough syrup formulation, 1g of extracts of *Allium cepa*, *Ocimum sanctum*, and Mentha *piperita* ext. were added in simple sugar syrup[20] (Table-1). The simple sugar syrup with 66.7% w/w (as per I.P) sugar concentration will act as an antimicrobial medium by itself due the high osmotic pressure[20]. The same formulation is prepared with pectin I.P as suspending agent for diabetic category individuals.

2.4. HPTLC fingerprint analysis for the selected herbals

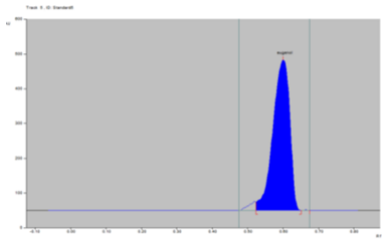
2.4.1. Ocimum sanctum (Tulsi) [8,21,22,23]

Source: Dried leaves of Ocimum tenuiflorum L Family: Lamiaceae
Sample preparation: 1g of powdered sample was mixed with 10 ml of methanol and then it is sonicated for 10 min. The mixtures were then centrifugated to obtain a clear supernatant, which is used as test solution and the final sample concentration after dilution: 2mcg/µl
Reference substances[14:] 1mg/ ml of eugenol (standard solution A) was dissolved in methanol and different amount of these were loaded into TLC plate.

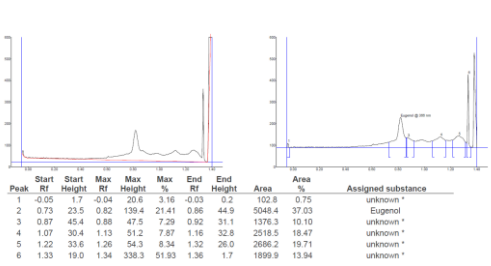
Stationary phase: HPTLC Si 60 F254, Mobile phase: Toluene, ethyl acetate 93:7 (v/v) for 30 min, Application: 2 microliters of standard solution, and 2 microliters of sample solution were applied on an 8 mm wide plate. Development: Saturation time: 20 min with saturation pad, Relative humidity: 33%,Visualization: UV 366 nm, Marker selected: Eugenol Rf value: 0.83. The results are reported in Fig.1**,** Fig.4, Table 2.



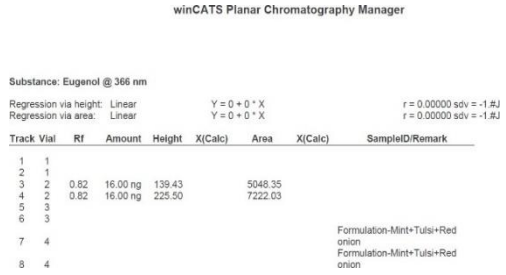
HPTLC finger print of *Ocimum sanctum* leaf extract



HPTLC finger print of eugenol Rf value: 0.82



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.05	1.7	-0.04	20.6	3.16	-0.03	0.2	102.8	0.75	unknown *
2	0.73	23.5	0.82	139.4	21.41	0.86	44.9	5048.4	37.03	Eugenol
3	0.87	45.4	0.88	47.5	7.29	0.92	31.1	1376.3	10.10	unknown *
4	1.07	30.4	1.13	51.2	7.87	1.16	32.8	2518.5	18.47	unknown *
5	1.22	33.6	1.26	54.3	8.34	1.32	26.0	2686.2	19.71	unknown *
6	1.33	19.0	1.34	338.3	51.93	1.36	1.7	1899.9	13.94	unknown *



winCATS Planar Chromatography Manager

Substance: Eugenol @ 366 nm

Regression via height: Linear Y = 0 + 0 * X r = 0.00000 sdv = -1.#J
Regression via area: Linear Y = 0 + 0 * X r = 0.00000 sdv = -1.#J

Track	Vial	Rf	Amount	Height	X(Calc)	Area	X(Calc)	SampleID/Remark
1	1							
2	1							
3	2	0.82	16.00 ng	139.43		5048.35		
4	2	0.82	16.00 ng	225.50		7222.03		
5	3							
6	3							
7	4							Formulation-Mint+Tulsi+Red onion
8	4							Formulation-Mint+Tulsi+Red onion

Fig 1: HPTLC analysis of *Ocimum sanctum* leaf extract

2.4.2. Mentha piperita [15,16,17,18]

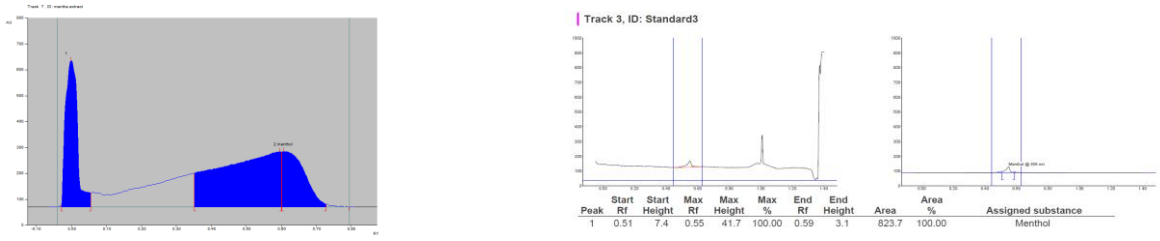
Source: Dried leaves of *Mentha piperita*, Family: Labiatae

Sample preparation: 1g of powdered sample is mixed with 10 ml of methanol and then it is sonicated for 10 min.

The mixtures are then centrifugated to obtain a clear supernatant, which is used as test solution and the final sample concentration after dilution: 2mcg/µl

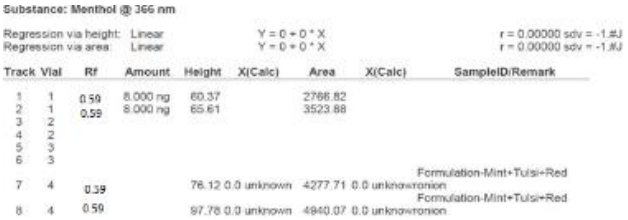
Reference substance: 1mg/ ml of menthol was dissolved in methanol and different amount of these will be loaded into TLC plate,

Stationary phase: HPTLC Si gel 60 F254, Mobile phase: Toluene: ethyl acetate 93:7 (v/v), Application: Different aliquots of standard solution, and of sample solution will be applied on an 8 mm wide plate and ascending development through twin trough chamber, Visualization: UV 366 nm, Marker selected: menthol (Rf value: 0.59). The results are reported in Fig.2, Fig.4, Table 2.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.51	7.4	0.55	41.7	100.00	0.59	3.1	823.7	100.00	Menthol

HPTLC finger print of *Mentha piperita* leaf extract
with the marker menthol (Rf value: 0.59)



Substance: Menthol @ 366 nm

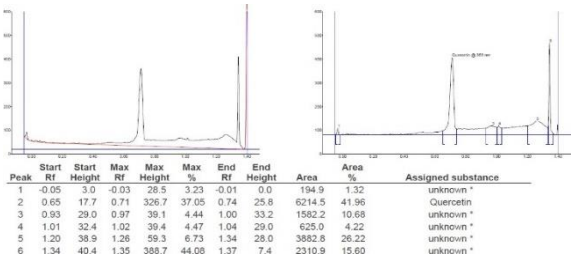
Regression via height: Linear Y = 0 + 0 * X r = 0.00000 sdv = -1.#J
Regression via area: Linear Y = 0 + 0 * X r = 0.00000 sdv = -1.#J

Track	Vial	Rf	Amount	Height	X(Calc)	Area	X(Calc)	SampleID/Remark
1	1	0.59	8.000 ng	60.37		2766.82		
2	1	0.59	8.000 ng	65.61		3523.88		
3	2							
4	2							
5	3							
6	3							
7	4	0.59		76.12	0.0 unknown	4277.71	0.0 unknown	Formulation-Mint+Tulsi+Red onion
8	4	0.59		97.76	0.0 unknown	4940.07	0.0 unknown	Formulation-Mint+Tulsi+Red onion

Fig 2: HPTLC analysis of *Mentha piperita* leaf extract

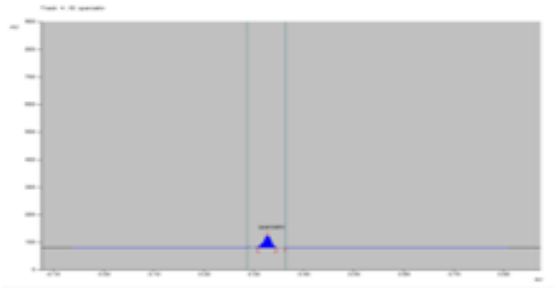
2.4.3. Allium cepa [19]

Source: Dried leaves of *Allium cepa* L Family: Liliaceae
Sample preparation: 1g of powdered sample is mixed with 10 ml of methanol and then it is sonicated for 10 min. The mixtures are then centrifugated to obtain a clear supernatant, which is used as test solution. Final sample concentration after dilution: 2mcg/µl.
Reference substance: 1mg/ ml of menthol was dissolved in methanol and different amount of these will be loaded into TLC plate, Stationary phase: HPTLC Si gel 60 F254, Mobile phase: Toluene, ethyl acetate: formic acid - 5:4:1 (v/v), Application: Different aliquots of standard solution, and of sample solution will be applied on an 8 mm wide plate and ascending development through twin trough chamber, Visualization: UV 366 nm, Marker selected: quercetin (Rf value: 0.73). The results are reported in Fig.3**,** Fig.4, Table 2.

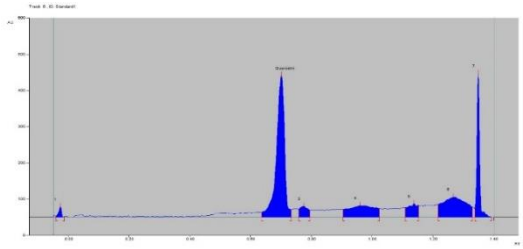


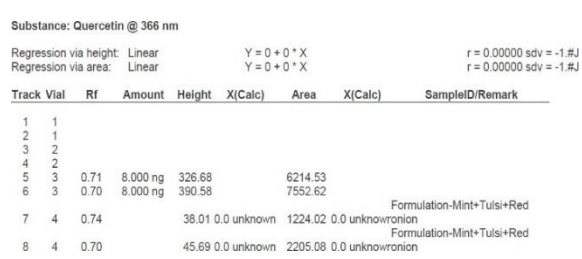
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.05	3.0	-0.03	28.5	3.23	-0.01	0.0	194.9	1.32	unknown *
2	0.65	17.7	0.71	326.7	37.05	0.74	25.8	6214.5	41.96	Quercetin
3	0.93	29.0	0.97	39.1	4.44	1.00	33.2	1582.2	10.68	unknown *
4	1.01	32.4	1.02	39.4	4.47	1.04	29.0	625.0	4.22	unknown *
5	1.20	38.9	1.26	59.3	6.73	1.34	28.0	3882.8	26.22	unknown *
6	1.34	40.4	1.35	388.7	44.08	1.37	7.4	2310.9	15.60	unknown *

HPTLC finger print of *Allium cepa* extract



HPTLC finger print of quercetin (Rf value 0.73)





Substance: Quercetin @ 366 nm

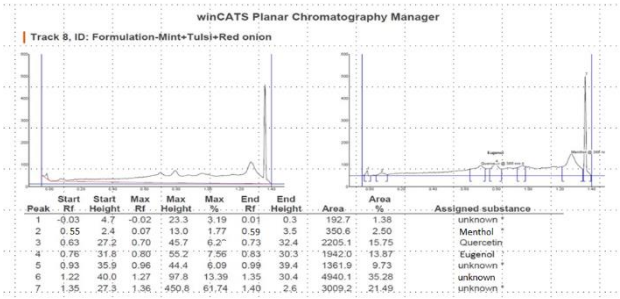
Regression via height: Linear Y = 0 + 0 * X r = 0.00000 sdv = -1.#J
Regression via area: Linear Y = 0 + 0 * X r = 0.00000 sdv = -1.#J

Track	Vial	Rf	Amount	Height	X(Calc)	Area	X(Calc)	SampleID/Remark
1	1							
2	1							
3	2							
4	2							
5	3	0.71	8.000 ng	326.68		6214.53		
6	3	0.70	8.000 ng	390.58		7552.62		
7	4	0.74		38.01	0.0 unknown	1224.02	0.0 unknown	Formulation-Mint+Tulsi+Red onion
8	4	0.70		45.69	0.0 unknown	2205.08	0.0 unknown	Formulation-Mint+Tulsi+Red onion

Fig 3: HPTLC analysis of *Allium cepa* extract

Table 2: HPTLC quantitation of the herbs selected

Herbs selected	Marker selected	Rf value	% Yield w/w	Amount of marker present in the herb extract mcg (w/w)	Quantity Percentage (%)
Ocinum sanctum	Eugenol	0.83	19.0	0.008	0.8
Mentha piperita	Menthol	0.59	24.0	0.008	0.8
Allium cepa	Quercetin	0.73	8.35	0.004	0.4



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.03	4.7	-0.02	23.3	3.19	0.01	0.3	192.7	1.38	unknown *
2	0.55	2.4	0.07	13.0	1.77	0.59	3.5	350.6	2.50	Menthol *
3	0.63	27.2	0.70	45.7	6.2"	0.73	32.4	2205.1	15.75	Quercetin
4	0.76	31.8	0.80	55.2	7.56	0.83	30.3	1942.0	13.87	Eugenol
5	0.93	35.9	0.96	44.4	6.09	0.99	39.4	1361.9	9.73	unknown *
6	1.22	40.0	1.27	97.8	13.39	1.35	30.4	4940.1	35.28	unknown
7	1.35	27.3	1.36	450.8	61.74	1.40	2.6	3009.2	21.49	unknown *

Fig 4: HPTLC analysis of the phytopharmaceutical formulation

2.5. Anti-microbial activity studies[24]

The culture of *E. coli* and *Staphylococcus aures* were used for the study. The collected bacteria were subcultured on sterile nutrient agar slants prepared in test tubes and incubated at 37^{o}C±0.5^{o}C for 24h.The nutrient agar media was heated to liquify, and cooled to 40^{o}C. The sterile media was then inoculated with each bacterial culture separately. These sterile inoculated media were poured into previously sterilized petridishes aseptically.

The concentration (1mg/ml) of the crude drugs present in the phytopharmaceutical formulation were prepared in dimethyl formamide. Small discs of 6mm diameter were cut from Whatman filter paper no:1 and sterilized. Ten microlitres of 1mg/ml crude drug extracts were added individually to sterile discs using a micropipette and then allowed to dry. All the petridishes were incubated at 37^{o}C ± 0.5^{o}C for 24h. Azithromycin (10mcg/ml) was used as the standard. The results are reported in Fig.5**,** Table 3.

Table 3: Zone of inhibition of the extracts included in the phytopharmaceutical formulation

Microorganism used	Zone of inhibition [Mean± SD (m.m)]					
	MLE l0mg/ml	TLE l0mg/ml	ACE l0mg/ml	AZ 1mg/ml	SS l0mg/ml	PPF 1mg/ml
E. coli (gram -ve)	9.4±0.45*	7.5±0.23	8.6±0.64	18±0.052**	8±0.102	11.8 ± 0.5
Staph.aures (gram+ve)	9.5±0.2	8.0±0.58	9.6±0.26	20.6 ±0.6**	9.2±0.13	10.6±0.4

n=3, student t test, * p value < 0.1, ** p value < 0.01, Concentration of the extracts used: (10mg/ml)
MLE: Mentha leaf extract, TLE: Tulsi leaf extract, ACE: *Allium cepa* extract, AZ: Azithromycin
SS: Sugar syrup 66.7% w/v, PPF: Phytopharmaceutical Formulation (MLE+TLE+ACE+SS)

Fig 5: Zone of inhibition of the extracts included in the phytopharmaceutical formulation using *E. coli* and *S. aureus*

2.6. Immunostimulant activity studies[25]

Candida albicans was used for the immunostimulant activity screening study. Human blood (2-3 drops) was taken from a healthy volunteer with prior informed consent and documented with the institutional ethics committee (IEC/PPG/11/22). The slide with the blood collected was kept on a cotton pad in a sterile petridish and incubated at 37ºC for 25 min. After incubation the clot was removed very gently and the slide was slowly drained with sterile normal saline taking care not to wash the adhered neutrophilis. The slide was flooded with predetermined concentration of PPF (MLE+TLE+ACE+SS) at 50 &100 mg/ml, incubated at 37ºC for 15 min and flooded with a suspension of Candida albicans in Hank's balanced salt solution and human serum and incubated at 37ºC for Ih.

After this, the slide was drained, fixed with methanol and stained with Giesma stain. The mean number of phagocytosed cells on the slide was determined microscopically for 100 granulocytes. This number was taken as the phagocytic index (PI) and was compared with the basal phagocytic index of control. The percentage immunostimulation was calculated by using the equation. The results are tabulated in Table 4, Fig.6.

$$\% \text{ immunostimulation} = PI_{(T)}\text{-}P\ I_{(c)} / P\ I\ (C) \times 100$$

$PI_{(T)}$ – Phagocytic index of test, $PI_{(C)}$ – Phagocytic index of control

Table-4. Immunostimulant activity studies

Drug Groups (n)	Dose mg/ml	Phagocytic index	% Immunostimulation
PPF	50	42 ± 0.816	64 ± 3.26
PPF	100	48 ± 0.47	82 ± 0.87**
Control (normal saline)	-	24 ± 0.8164	-

n=5 p value < 0.01**, < 0.05* PPF: Phytopharmaceutical Formulation (MLE+TLE+ACE+SS)
PPF: Phytopharmaceutical Formulation (MLE+TLE+ACE+SS)
MLE: Mentha leaf extract, TLE: Tulsi leaf extract, ACE: *Allium cepa* extract

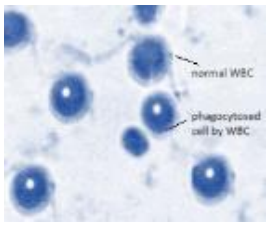


Fig 6: Phagocytosis of *Candida albicans* by WBC

Table 5: HERBOVIGILANCE STUDIES

HERB NAME	PHARMACOKINETIC INTERACTIONS	PHARMACODYNAMIC INTERACTIONS
Tulsi[9,11] *Ocimum sanctum*	Tulsi has no major potential pharmacokinetic interactions The major active compounds in tulsi (*Ocimum sanctum*), such as eugenol, carvacrol, and linalool, inhibit CYP1A1&1B1, thereby preventing the conversion of the procarcinogen Benzo [a] pyrene to toxic diolepoxide[15]. Some studies showed that antidiabetics[19] drugs along with tulsi result in excess lowering of blood sugar levels.	Use of tulsi along with anti-coagulant Clopidogrel increases the risk of bleeding. Tulsi has the property to thin the blood; hence it should not be taken along with other anti-clotting medications. Also, patients who are taking blood-thinning medications like warfarin and heparin should restrict the consumption of Tulsi which may intensify the blood thinning properties of the prescribed drugs. Benzo[a]pyrene (BAP) is a polycyclic aromatic hydrocarbon (PAH) which is proved as an environmental pollutant produced from the incomplete combustion of coal tar, tobacco smoke, automobile exhaust, grilled meat etc. Diol epoxides are DNA adducts that mutate the p53 tumor suppressor gene, results in cancer[16]. Also, repeated exposure to Benzo [a] pyrene may lead to darkening and thickening of skin, appearance of pimples etc.
Mentha [16,17,18] *Mentha piperita*	CYP3A4 (inhibition)	Felodipine effect will be enhanced
Red onion[12] *Allium cepa*	Quercetin is a flavonoid present in onion possibly interact with felodipine by preventing the breakdown of the drug into an inactive form and thereby increase the blood level of the drug. Red onion contains compounds called diallyl disulphide which can cause allergy symptoms like asthma, nasal congestion and contact dermatitis.	Felodipine effect will be enhanced May interact with anti-allergic drugs

3. DISCUSSION

The HPTLC of tulsi leaf extract was performed & the developed plates were visualized in UV 366nm. Eugenol at Rf value: 0.83 was used as the marker in the evaluation. The mentha leaf extract showed menthol at Rf value: 0.59 and used as the marker. Quercetin at Rf value: 0.73 was used as the marker for *Allium cepa*. The HPTLC analysis of the phytopharmaceutical formulation indicated that, an amount of 0.008 mcgs of eugenol and quercetin, 0.004 mcg of menthol are present per mcg of *Ocimum sanctum*, *Allium cepa* and *Mentha piperita* extracts respectively.

The herbovigilance reviews (Table 5) indicated that, the enzymes CYP1B1 specially[18,19] will get inhibited by eugenol, linalool etc present in tulsi and it cannot further activate benzo[a]pyrene and it will be excreted as such without converting into harmful carcinogen. Felodipine effect will be enhanced if taken together with *Mentha piperita* by the CYP3A4 inhibition by Mentha extracts [16,17,18]. The flavonoid quercetin present in *A. cepa* possibly interact with felodipine by preventing the breakdown of the drug into an inactive form and thereby increase the blood level of the drug.

Onion contains compounds called diallyl disulphide which can cause allergy symptoms like asthma, nasal congestion and contact dermatitis.

The antimicrobial activity studies showed, zone of inhibition of 11.8 mm and 10.6mm for *E. coli* (gram-ve) and *Staph.aures* (gram +ve) respectively by PPF. The percentage immunostimulation was found to be 64% and 82% for the PPF at the dose of 50mg/ml and100 mg/ml respectively.

4. CONCLUSION

The present work highlights the HPTLC evaluation of an expectorant phytopharmaceutical prepared from three herbs namely *Ocimum sanctum*, *Mentha piperita* and *Allium cepa* as per the guidelines of Indian Pharmacopoeia Commission, Ministry of Health and family welfare, Govt. of India. Individual herb and the combined HPTLC evaluation procedures were carried out and documented the results. Four therapeutic markers representing the three herbs included in the phytopharmaceutical formulation are eugenol, menthol and quercetin for the herbs *Ocimum sanctum*, *Mentha piperita* and *Allium cepa* respectively. The developed HPTLC method is found to be reproducible and accurate.

The PPF showed, effective antimicrobial activity against gram +ve and gram – ve bacteria and also produced considerable phagocytic index in the invitro immunostimulation studies with *Candida albicans*.

The herbovigillance protocols framed for the patients under cyclosporine, anti-diabetic drugs, anti-coagulants, anti-allergic drugs etc., for monitoring carefully the action of PPF along with conventional drugs. These protocols may give good information about various herb-drug interactions for the patients during the post covid-19 period.

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