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APPLICATION OF RESPONSE SURFACE METHODOLOGY FOR ANTIOXIDANT EVALUATION OF *TANACETUM UMBELLIFERUM* BOISS; TARGETING GOUT AND HYPERURICEMIA

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Abstract:
Oxidative degeneration of cells and tissues is the common concern of scientific research and debate in medical sciences round the globe. Oxidative stress in body is reflected by generation of reactive oxygen species (ROS) that are free radicals with least count of unpaired electrons having high reactivity. Imbalance between biological system and ROS is responsible for degeneration of tissues and became toxic to induce misfolding of protein, activation of glia cell, mitochondrial malfunction and apoptosis of cell. One of the susceptible mechanisms for degenerative changes of joints is generation of ROS. Traditionally claimed *Tanacetum umbelliferum* is an enduring member of the "Asteraceae" family, and defeats the manifestations of arthritic and gouty conditions where one of the most probable underlying mechanisms is free radical scavenging (FRS). The current research was planned to find out FRS capacity of *T. umbelliferum* using *in vitro* antioxidant activity by DPPH assay and effect of the different solvents (ethanol, chloroform, 1 butanol, n-hexane) on the extraction of bioactive chemicals. The experimental design was determined at optimum level using response surface methodology technique. Phytochemical screening declared the presence of alkaloids, flavonoids, phenols and glycosides that are suspected to be responsible for antioxidant potential. The HE crude extract demonstrated more activity than the fractions, at 3.1 mg/mL, the percentage inhibition was determined as 99.9, 94, 88.4, 80 and 71% for ascorbic acid, crude HE extract, and 1-butanol, chloroform, n-hexane fractions respectively, where the tapering of dose sequentially reduced the inhibition percentage. As lower IC_{50} reflects higher DPPH scavenging activity. Therefore, it is deducted that polar phytochemicals are potent free radical scavengers and being constituted with these phytochemicals *T. umbelliferum* is proven to be the effective, safe and potent option for management of arthritic disorders such as gout and hyperuricemia.

Index Terms: Antioxidant, Arthritis, Cell degeneration, Gout, Phytochemical analysis, ROS, Solvent fractions, *T. umbelliferum*.

1 INTRODUCTION

This Based upon gender (women's affected rate is two to three times added frequently than man), age (incidence for latest RA detect peaks into 6th decade for life), the considered patient population (Arthritis rate varies as of south toward north & may be elevated in city than countryside domain) by incidence scale as of 0.4% to 1.3% of inhabitants, one of majority common chronic

inflammatory illnesses is arthritis (Chen L et al., 2021). Oxidative stress created by reactive oxygen species is one of thematic causes of joint destruction. ROS are results of aerobic respiration in cells (Said, M.S., et al. 2022).

Approximately 40% of patients present extra-articular symptoms, with the onset occurring at any stage in the disease and with the likelihood of occurrence equal amongst both men and women (Johar, U. and Q. Tahir, 2022).

The prestigious saying of Hippocrates "Let prescription be your food and food your medicine shed the light on the meaning of natural medications. The use of medicinal plants is essentially just about as old as human existence. Most of the countries like South America, Asia, and Africa have a couple of collections of plants to utilize and counter different diseases as treatment option. That is the clarification World Health Organization (WHO) proclaimed that the pivotal clinical benefits for the 80% individuals all through the planet contain the natural medications. Curative plants are utilized as an elective source to manage diverse clinical issues. Various investigations archive numerous organic impacts of *Tanacetum umbelliferum* and among others, this plant is utilized in conventional medication for the treatment of joint ailments such as gout and arthritis.

T. umbelliferum is an enduring zest of the "Asteraceae" family, inhabited to Europe. As shown by the literature assessment the principal oil of *T. umbelliferum* is segregated into four phytochemicals; 1,8-cineole, trans-thujone, camphor and myrtenol. Moreover, seven chemo types of *T. umbelliferum* are recognized i.e. α-thujone, β thujone, camphor, cysteinyl acidic acid determination/ chrysanthenol, chrysanthemum, artemisia ketone/artemisia alcohol, and 1,8- cineole (Heluta V et al., 2010). Many plants are found to exhibit marvelous potential of scavenging the free oxygen radicals therefore, possess excellent antioxidant activity to protect the articulations from degeneration. The lacking is experimental evidence of underlying mechanism and identification of core phytochemical responsible for particular mechanism. Therefore, the present study focused on in vitro antioxidant activity of *T. umbelliferum* hydroethanolic extract and its various fractions.

2. Material and Methods

2.1 Extraction of Experimental Plant

T. umbelliferum was collected and identified from Botany Department; The Islamia University of Bahawalpur the voucher number was "Ref. No. 39/ Botany". The powdered plant material 500g was extracted by maceration in hydroethanolic solvent (70: 30). The solute to solvent ratio for mixture was 1:10 w/v. After soaking for 3 days the mixture was filtered finally with whatman filters paper. Filtrate was evaporated using rotary evaporator at 50-60 °C the consequent concentrate was then collected in sealed container and kept in at 04±1°C for further use. Percentage yield was calculated for obtained extract (Chhetri SBB et al., 2020).

2.2 Phytochemical Analysis

Standard techniques were used to determine the presence of carbohydrates, amino acids, proteins, lipids, saponins, flavonoids, alkaloids, tannins, and phenols in the plant (Tiwari S et al., 2020).

2.3 Fractions Preparation

Fractions of crude hydroethanolic extract were prepared using different solvents. Solvent selection was polarity based such as least, medium and highly polar to isolate metabolites likely to dissolve in different polarity. N-hexane, 1-butanole, chloroform and hydroethanolic solvents were used for fractionation (Truong DH et al 2019).

Calculated the percentage yield of each fraction using formula:

% yield= weight of fraction obtained/weight of crud extract used x 100.

2.4 Antioxidant Activity

2, 2-diphenyl-1-picryl-hydrezyl (DPPH) was utilized as free radical agent in order to experiment the antioxidant potential. The experimental design and dosage calculation with reference to temperature

and DPPH concentration was calculated using Response Surface Methodology technique, for that Design Expert Software Model 13 was implemented to optimize the experimental conditions. 17 runs were predicted against three factors i.e. concentration of test samples mg/ml, temperature ℃ and DPPH concentration mg/20ml, out of which four highly predicted significant runs were selected i.e. at 3.1, 2.5, 1.5 and 0.5 mg/ml concentrations of test samples where temperature and DPPH concentration was taken as constant i.e. 37℃ and 1.4mg/20ml respectively as final working design of experiment. Ascorbic acid was used as standard and methanol as negative control. Among 100µl of total volume, 90µl of DPPH solution along with 10µl testing sample of drug was taken in 96-well microplate following mixing of the contents by thorough pipetting. After that the solution taken in the 96-well microplate was incubated at 37℃ for half hour and 517nm was the wavelength to take the absorbance by using microplate reader (HT BioTEk USA). Readings were compared with the standard utilized and all observations were obtained in thrice of the readings (Tokoudagba KJMD and Gbaguidi FA, 2022). The programming was utilized to ascertain the IC_{50} value was Ez-fit-5 Perrella Scientic Inc., Amherst USA. Decrease in absorbance demonstrated expanded radical scavenging action that was obtained via underlying equation:

$$\%I = \frac{Ac - At}{At} \times 100$$

Where; Ac=Abs. of control, At=Abs. of test

2.5 Statistical Analysis

The data was analyzed using Response surface methodology and Design Expert Software Model 13 was implemented and expressed as Mean with Standard Error of Mean. ANOVA was used for analysis.

3. Results and Discussion

3.1 Phytochemical Investigation

The phytochemical investigation on the aqueous ethanolic extract of *T. umbeliferum* roots was performed to determine the presence of phytomolecules, shows the presence of carbohydrates, amino acids, lipids, saponins, flavonoids, alkaloids, tannins, and phenols and absence of proteins in extract.

3.2 Percentage Yield of Test Samples (Extract and Fractions)

The plant powder subjected to extraction in hydroethanolic solvent generated a semisolid crude extract. The percentage yield of this crude extract was calculated using formula given above. Similarly the obtained yield of crude HE extract was further subjected to dissolution for collecting fractions of three different solvents i.e. n-hexane, 1-butanol and chloroform. The percentage yield for each extraction was calculated and given in table 1.

TABLE.1: Percentage Yield of Crude extract of *T. umbelliferum* and its Fractions

Test Sample	Solute in gram	Obtained yield (g)	Percentage yield (%)
HE extract	500	30g	06
nH	20	0.259	1.295
1B	20	0.345	1.725
Ch	20	0.484	2.4

HE (hydro-ethanol); nH (n-hexane); Ch (chloroform); 1B (1-butanol)

3.3 Results of Antioxidant activity of Test Samples (Extract and Fractions)

Oxidative stress levels can be controlled by administrating antioxidants as primary treatment. Antioxidants are the agents used to eradicate ROS, that are responsible for degenerative changes in arthritic disorders. The efficiency of antioxidants in averting additional degeneration is not dependent only on disease but also on sex, age and ethnicity. Antioxident activity of *T.*

umbelliferum is performed in this study. The antioxidant effect of test sample is given below in table 2.

TABLE. 2: Percentage of inhibition of DPPH and IC50 for crud extract and Fractions of plant roots at different concentrations (mg/mL) compared with standard.

Fractions with different solvents	Test Samples	Conc. mg/mL				
		3.1	2.5	1.5	0.5	
		Percentage Inhibition (%)				IC_{50}
	Ascorbic acid	99.9 ± .0296	98± .0296	96 ± .0512	93± .1067	0.01
	HE	94± .1067	91± .0596	88± .1025	85± .0925	0.04
	1B	88.4± .0296	84± .1035	80± .0925	79± 11.3825	0.14
	Ch	80± .0745	76± .8112	73± .0512	68± .0412	3.10
	nH	71± .7112	67± .7112	63± .0312	60± .0296	11.10

HE (hydro-ethanol); nH (n-hexane); Ch (chloroform); 1B (1-butanol)

Response Summary

ANOVA for Quadratic model of each fraction

TABLE. 3. Various Runs of selected three factors and their predicted values of % inhibition

Factor 1 A:test sample C... mg/ml	Factor 2 B:Tempreture C	Factor 3 C:DPPH mg/20ml	Response 1 P.I of HE %	Response 2 PI of B.1 %	Response 3 P.I of Chloroform %	Response 4 P.I of N.h %
-0.181793	37	1.4	50	40	29	19
1.5	37	1.06364	68	57	48	43
1.5	37	1.4	88	80	73	63
2.5	37	1.4	91	84	76	67
1.5	37	1.73636	76.76	66.6	53.43	48
0.5	37	1.4	85	79	68	60
2.5	39	1.6	81	73	65.54	59
0.5	35	1.2	45	38	25	13
1.5	33.6364	1.4	54	44	33.87	27
2.5	35	1.6	65	53.65	45	39
1.5	37	1.4	72	62	50	45
3.18179	37	1.4	94	88.4	80.34	71
1.5	37	1.4	77	67	58.32	52
2.5	35	1.2	63	49.78	41	36.65
1.5	40.3636	1.4	83	76	69	62
0.5	35	1.6	57.77	45	37	33
0.5	39	1.2	79	69	63.21	56

TABLE. 4. Response 1. ANOVA for Quadratic model-Percentage inhibitions of hydroethanolic extract (HE)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2181.02	3	727.01	7.43	0.0038	significant
A-test sample Concentration	778.75	1	778.75	7.96	0.0144	
B-Tempreture	1317.13	1	1317.13	13.47	0.0028	
C-DPPH	21.97	1	21.97	0.2246	0.6434	
Residual	1271.33	13	97.79			
Lack of Fit	1137.33	11	103.39	1.54	0.4581	not significant
Pure Error	134.00	2	67.00			
Cor Total	3452.35	16				

The Model F-value of 7.43 implies the model is significant. There is only a 0.38% chance that an F-value this large could occur due to noise.
P-values less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.
The Lack of Fit F-value of 1.54 implies the Lack of Fit is not significant relative to the pure error. There is a 45.81% chance that a Lack of Fit F-value this large could occur due to noise.

Non-significant lack of fit is good -- we want the model to fit.

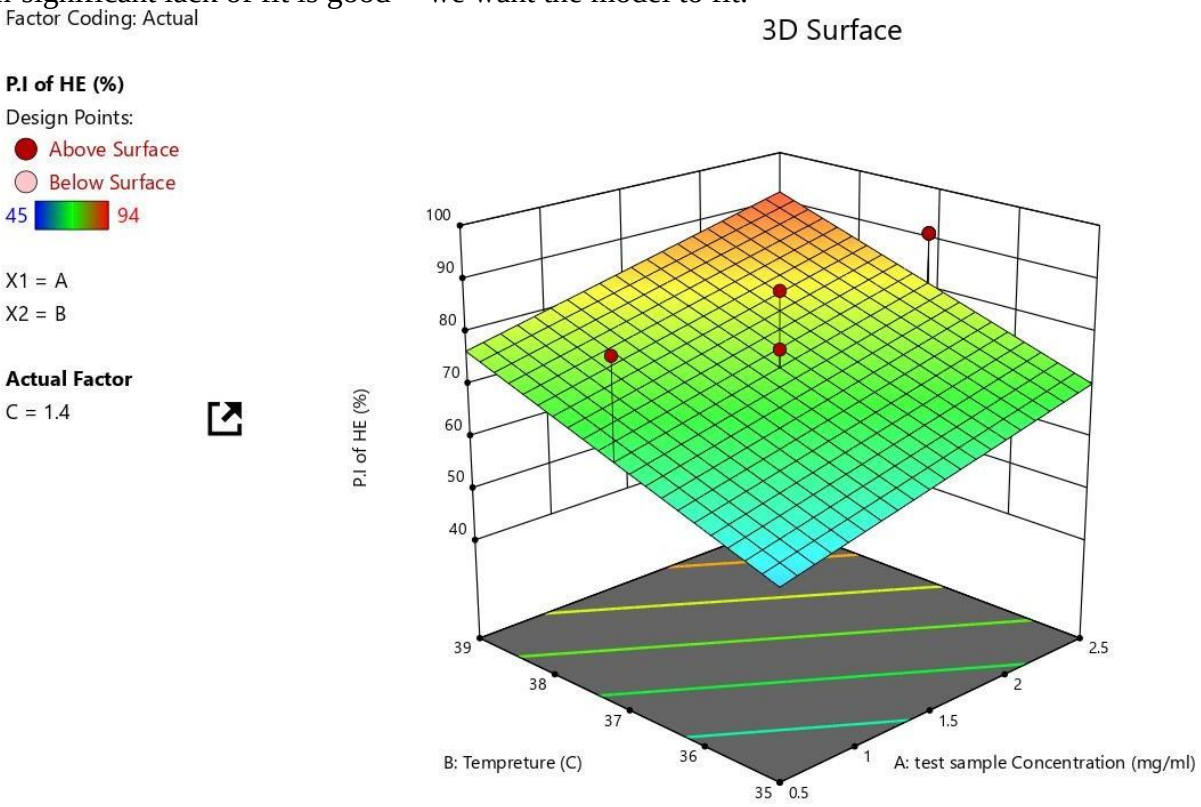

Fig. 1. 3D Surface Graph Percentage inhibitions of hydroethanolic extract (HE)

TABLE. 5. Response 2: ANOVA for Quadratic model-Percentage inhibitions of 1-Butanol (B.1)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2527.47	3	842.49	6.95	0.0049	significant
A-test sample Concentration	836.12	1	836.12	6.90	0.0209	
B-Tempreture	1608.83	1	1608.83	13.27	0.0030	
C-DPPH	19.24	1	19.24	0.1587	0.6968	
Residual	1575.72	13	121.21			
Lack of Fit	1403.06	11	127.55	1.48	0.4718	not significant
Pure Error	172.67	2	86.33			
Cor Total	4103.19	16				

The Model F-value of 6.95 implies the model is significant. There is only a 0.49% chance that an F-value this large could occur due to noise.
P-values less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are

many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.
The Lack of Fit F-value of 1.48 implies the Lack of Fit is not significant relative to the pure error. There is a 47.18% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

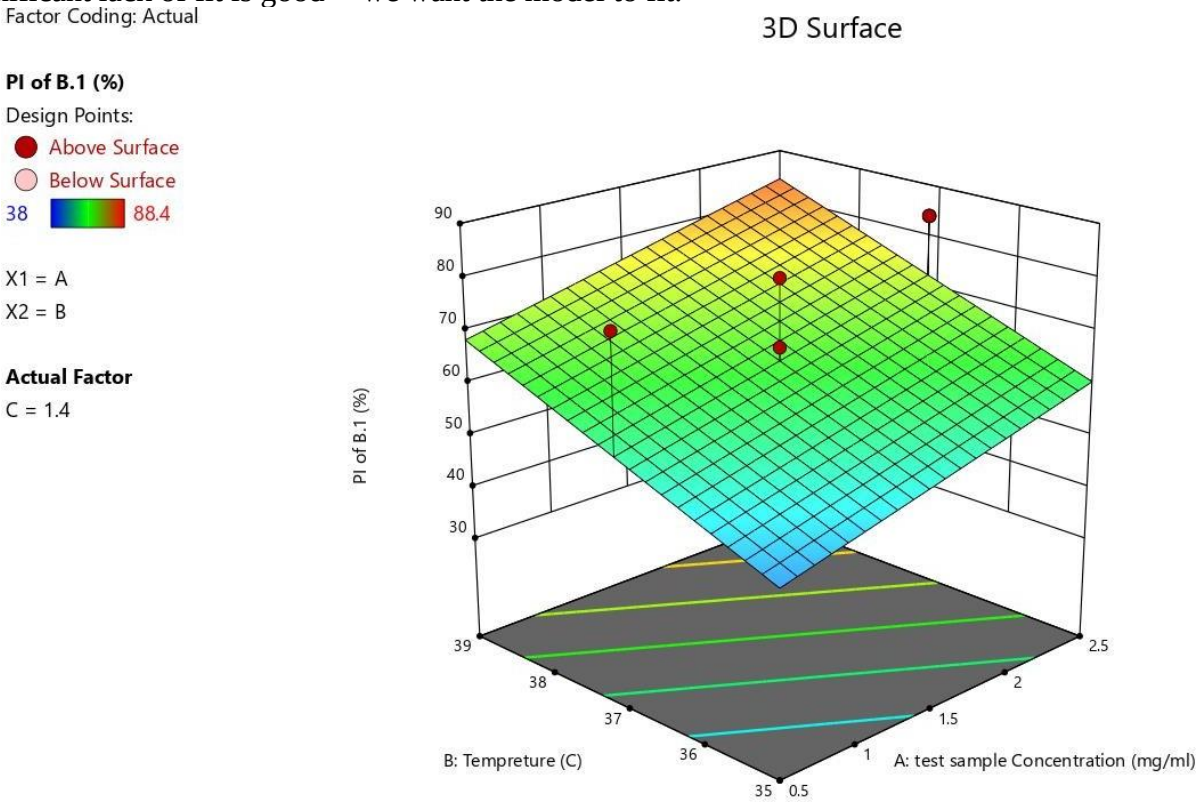

Fig. 2. 3D Surface Graph Percentage inhibitions of 1-Butanol (B.1)

TABLE. 6. Response 3: ANOVA for Quadratic model-Percentage inhibitions of Chloroform

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2981.16	3	993.72	7.70	0.0033	significant
A-test sample Concentration	1010.02	1	1010.02	7.83	0.0151	
B-Tempreture	1906.44	1	1906.44	14.77	0.0020	
C-DPPH	8.43	1	8.43	0.0654	0.8022	
Residual	1677.53	13	129.04			
Lack of Fit	1406.29	11	127.84	0.9427	0.6209	not significant
Pure Error	271.24	2	135.62			
Cor Total	4658.69	16				

The Model F-value of 7.70 implies the model is significant. There is only a 0.33% chance that an F-value this large could occur due to noise.
P-values less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.
The Lack of Fit F-value of 0.94 implies the Lack of Fit is not significant relative to the pure error. There is a 62.09% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

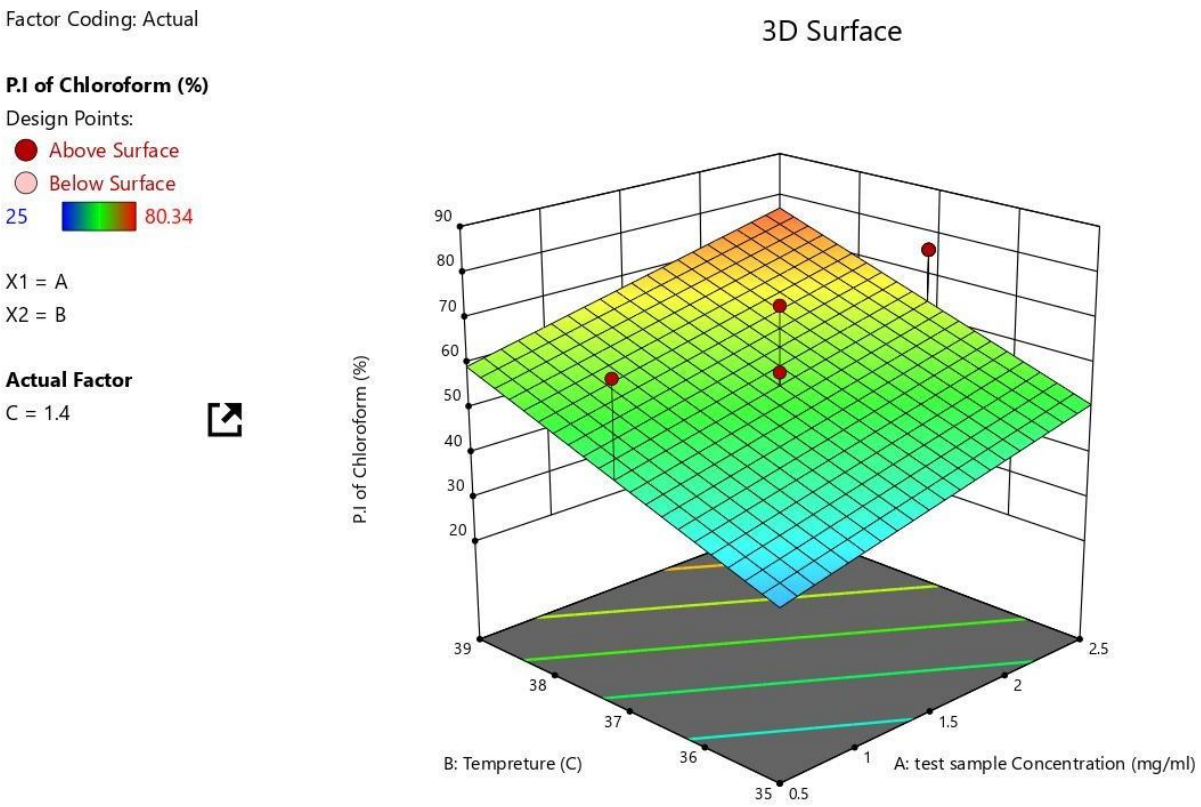

Fig. 3. 3D Surface Graph Percentage inhibitions of Chloroform

TABLE. 7: Response 4: ANOVA for Quadratic model-Percentage inhibitions of n-Hexane (Nh)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3058.78	3	1019.59	8.59	0.0021	significant
A-test sample Concentration	1105.36	1	1105.36	9.31	0.0093	
B-Tempreture	1855.66	1	1855.66	15.63	0.0017	
C-DPPH	20.18	1	20.18	0.1700	0.6868	
Residual	1543.66	13	118.74			
Lack of Fit	1378.99	11	125.36	1.52	0.4623	not significant
Pure Error	164.67	2	82.33			
Cor Total	4602.44	16				

The Model F-value of 8.59 implies the model is significant. There is only a 0.21% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The Lack of Fit F-value of 1.52 implies the Lack of Fit is not significant relative to the pure error. There is a 46.23% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

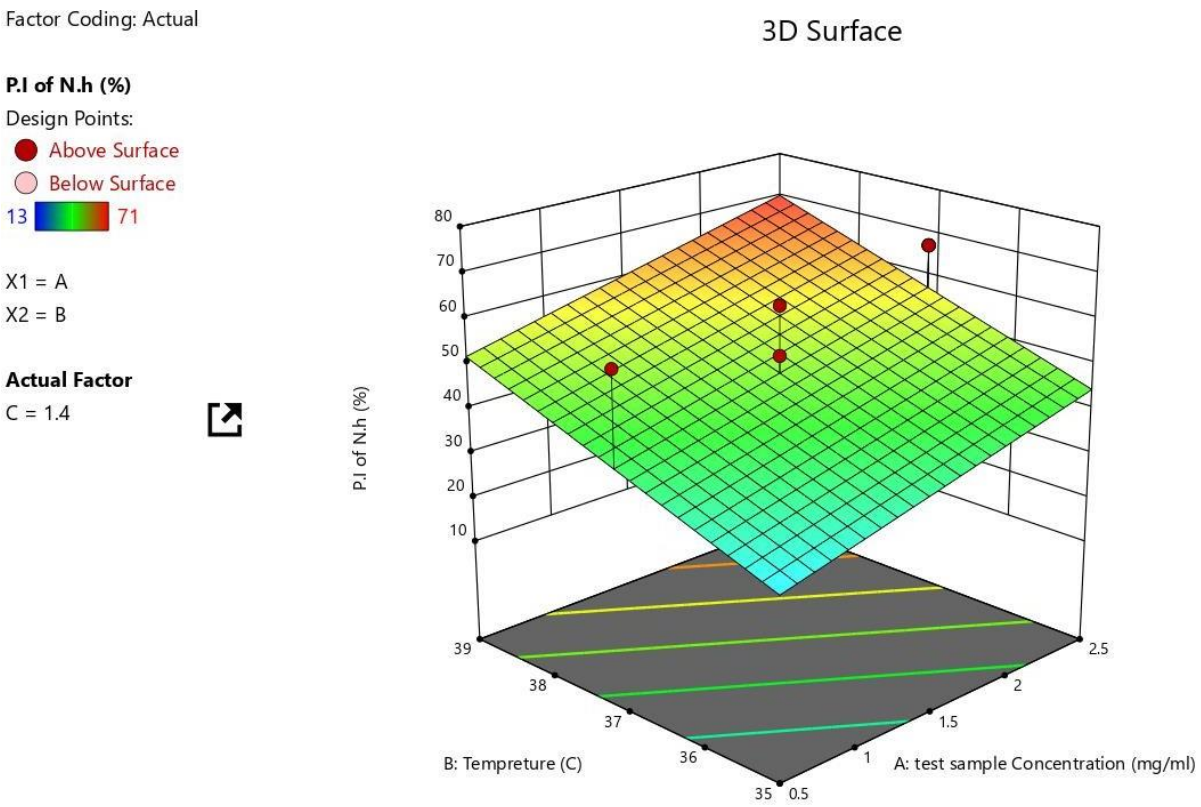

Fig. 4. 3D Surface Graph Percentage inhibitions of n-hexane (Nh)

3.5 Discussion

The study was designed to evaluate the antioxidant potential of *T. umbelliferum.* For this purpose the roots of plant were first extracted and then fractioned to lead towards the purification of target compounds moreover phytochemical analysis was performed to identify the active metabolites found in the tested plant extract. The information on the substance constituents of plants assists with screening for biological applications. The phenolic and flavonoids are broadly distributed metabolites in plants having antioxidant potential and have wide scope of implementation in clinical practices as natural medicine. The phytochemical trials affirm the occurrence of carbohydrates, terpenoids, glycosides, phenols, saponins, alkaloids, flavonoids and tannins whereas proteins and amino acids were not reported in extract of *T. umbelliferum.* It was proved to be supportive in regard of arthritis and gout as protein metabolites are one of the causative agents for such disorders and results in hyperuricemia.

Antioxidants play an important role in management of various disorders such as liver diseases, aging, cancer and inflammatory disorders, degenerative diseases and joint ailments because of their ability to scavenge free radicals (Shrestha D et al., 2020). To combat oxidative stress in the human body, these natural antioxidants can be synthesized as nutraceuticals. One method of determining antioxidant activity is to use the free radical DPPH (Veljkovic B et al., 2019). Table 2 shows that the activities of the test samples are more comparable to that of ascorbic acid. This is also logical, given that ascorbic acid is already pure, whereas various herbal extracts and fractions must still be treated to isolate the active components responsible for their antioxidant activity. This assay, on the other hand, could be utilized to help isolate putative antioxidant phytochemicals from this plant (Borges C et al., 2020).

The dose–response curve of DPPH radical scavenging activity of the aqueous ethanolic extract is shown off at 3.1mg, 2.5, 1.5 and 0.5mg/ml of *T. umbelliferum* and its fractions n-hexane, 1- butanol and chloroform at same dose compared with ascorbic acid. It was observed that the HE crude extract had higher activity compared to the fractions but yet less than ascorbic acid that is standard drug. At a concentration of 3.1 mg/mL, the scavenging activity of HE extract was determined as 94 % that decreases by 91%, 88% and 85% as dose tapered by 2.5mg, 1.5 mg and 0.5mg respectively

while that of the ascorbic acid was 99, 98, 96, 93% at the same concentrations. The results of HE extract were followed by 1-butanol with percentage inhibition of 88, 84, 80, 79 and chloroform fraction with 80, 76, 73, 68% respectively at same concentrations. Whereas, n-hexane fraction possess least significant activity with lowest values of % inhibition i.e. 71, 67, 63, 60% respectively at same concentrations. As lower IC_{50} reflects higher DPPH scavenging activity the crude extract possess highest radical scavenging potential compared to fractions. While among fractions the solvent having high polarity generated high antioxidant activity compared to nonpolar or least polar. Therefore, it is deducted that polar phytochemicals are more potent scavengers of reactive oxygen species. For crude hydroethanolic extract highest potential justifies that being a combination of medium to highest polar solvent it dissolves almost all bioactive metabolites that are responsible for boosting the therapeutic activities of plant and its parts. The results are supported by another study conducted on three medicinal extracted in different solvents where polar solvent extracts produced superior antioxidant activity compared to others (Kagambega W *et al.*, 2021). Therefore the lack of fit shows in significance that is supportive for significant results and directive towards the acceptance of research hypothesis i.e. *T. umbelliferum* possess significant antioxidant activity and is an alternative therapeutic option to manage arthritic disorders such as gout and hyperuricemia.

4. CONCLUSION

The current research study tends to be presumed that *T. umbelliferum* is a traditional plant possess noticeable *in vitro* antioxidant potential. By additional broad research to further isolate the particular polar phytochemicals, we can investigate the therapeutic worth and management role of *T. umbelliferum* in degenerative disorders like arthritis, gout, hyperuricemia etc at a commercial level.

5. Summery

Oxidative stress is created by nonenzymatic oxidation reaction during metabolism. Free radicals are key factors due to high reactivity and referred for production of reactive oxygen species. ROS in turn interfere the cellular processes and cause degeneration of cells and eventually of tissues. The destruction when occurs in articulated system it causes musculoskeletal diseases such as arthritis, rheumatism, gout etc. therefore, it is of interest to target the ROS for management of such disorders. Same was the aim of current study to evaluate scientifically the suspected and claimed natural sources that can scavenge the free radicals with in the body safely and effectively. T. umbelliferum is a traditional medicinal plant claimed to fit in to goal. Directed towards the isolation of particular antioxidant compound this study experimented the extraction and then fractionation of various metabolites of plant via polarity based solvents of low, medium and high polarity i.e. n-hexane, 1-butanole, chloroform and hydro-ethanol. The in vitro DPPH free radical scavenging assay was used to evaluate the antioxidant potential where RSM was implemented to optimize the experiment and statistical analysis via ANOVA. Using the four predicted concentrations of all test samples (fractions and crud hydroethanolic extract) percentage inhibition was highly scored by hydroethanolic extract that is comparable to positive control ascorbic acid, followed by 1-butanol, chloroform and n-hexane. The significance level increases by increasing the dose hat declared the dose dependent manner of antioxidant activity of test samples. Consequently it is declared that T. umbelliferum is a potent antioxidant for management of articular disorders especially gout as it is antihyperuricemic according to review of literature. Further compound isolation is the milestone towards future perspectives.

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8. CONFLICT OF INTERESTS DECLARATION

In relation to the research, authorship, and/or publication of this work, the authors disclosed no conflicts of interest.

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