

Biological Activities of Metal Complexes with Rutin and Bio-Conjugate of Citrus Extract

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Abstract The main aim of this study is to determine the changes occurred in the antioxidant capacity and UV-protection capacity of rutin with a different redox-potential metal cation. The complexes were characterized by UV-Visible and IR spectroscopy. Rutin belongs to the flavonols and it is a glycoside form of quercetin. Chelate formation is a result of interaction of rutin with metal ions. The chelation of metals can be crucial in the prevention of radical generation, which damages target biomolecules. The interactions between flavonoids and transition metal ions to form complexes that prevent the participation of metal ions in free radical generating processes, thus exhibit an anti-oxidant behavior, metal-flavonoid complexes various biological and pharmacological activities. The coordination of Rutin (Rut) with Rut-Zr, Rut-Mo, Rut-Zn, Rut-Cu, Rut-Ni, Rut-Fe, Cit-Zn, Cit-Mo, Cit-Zr, Cit-Cu, Cit-Fe, and Cit-Ni may simulate the catalyzed centre of the hyperoxide dismutase in the metal coordinate structure, thus displayed the DPPH, hydroxyl radical scavenging activity, metal chelating, and nitric oxide (NO) and superoxide dismutase (SOD) radical scavenging. Therefore, the metal flavonoid complexes are very helpful in discovery and development of new drugs.

Keywords Rutin, Metal Chelation, Flavonoid and Metabolites

1. Introduction

Flavonoids are a class of secondary plant metabolites

with significant antioxidant, chelating, and antimicrobial properties; hence, they have many beneficial effects on foods and health. Rutin belongs to the flavonols and is a glycoside form of quercetin. Rutin is naturally found in fruits and plants such as potatoes, onions, ginger, tomatoes, vegetables, and herbal drinks like tea (Om *et al.*, 2018). It has peroxy, hydroxyl, and superoxide radical scavenging effects (Sharma *et al.*, 2013). Its health promoting effects are also well acknowledged (Pan *et al.*, 2014; Wang *et al.*, 2015). Daily value of rutin is around 1.5–70 mg/kg, which varies as per nutritional habits and countries regulations (Kreft *et al.*, 1999; Nakamura *et al.*, 2000), very low aqueous solubility is the main drawbacks of rutin. Furthermore, it is not absorbed by intestinal membrane due to glycosylated structure with a disaccharide which makes it polar and large (Sharma *et al.*, 2013). In addition to improving chemical stability, odour and taste masking, colloidal dispersity, their active endocytosis form intestinal Payer's patches to increase oral bioavailability of rutin, making it a proper carrier for food fortification. Chelate formation is a result of interaction of rutin with metal ions. The chelation of metals can be crucial in the prevention of radical generation, which damages target biomolecules. Moreover, using natural chelators such as flavonoids is better than the synthetic ones due their toxicity effects. In the structure of several flavonoids (Fig1.0) are three potential coordination sites:

a) 5-hydroxy and 4-carbonyl group, **b)** 3-hydroxy and 4-carbonyl group, **c)** 3', 4'-hydroxy group in B ring.

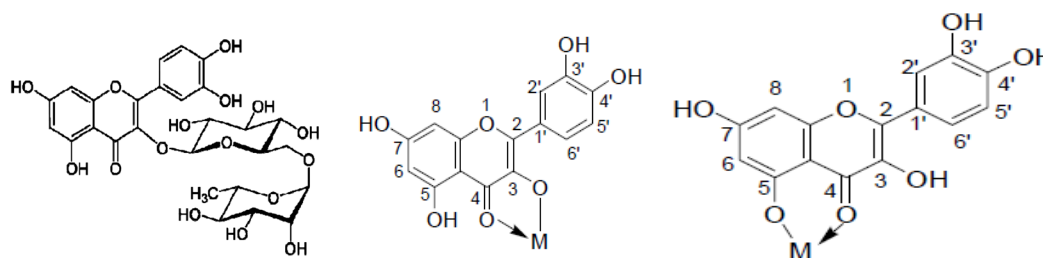


Figure 1. Structure of Rutin and Quercetin. (M= Metal)

The interaction of certain flavonoids with transition metals increases their activity and leads to the formation of flavonoid-metal complexes, which may act as scavenger and free radical. Effect of serum albumin on stability of flavonoid-metal complexes was studied and complex of rutin with iron (II) was found to be the most stable. The ability of flavonoid metal complexes to catalyze homolytic cleavage of hydrogen peroxide was also studied and rutin iron (II) complex was found to be relatively poor Fenton catalyst. The potential therapeutic benefits of this new antioxidant agent were studied using experimental model of pathological states associated with oxidative stress in vivo. It was found that pretreatment with rutin-iron complex protected against thio-acetamide induced hepato-toxicity as observed by a significant reduction in the elevated levels of serum enzymes and partial normalization of GSH/GSSG ratio, glutathione peroxidase II and glutathione reductase activity in mice liver. The results demonstrate that flavonoid-metal complexes possess effective free radical scavenger ability and have potent therapeutic benefits for the treatment of oxidative stress-related diseases and dysfunction. The antioxidant activity of flavonoids may involve their ability to form complex body iron in non-redox-active (Baccan *et al.* (2012).

Material Methods

1. Preparation of Metal Complexes of Rutin with Methanolic Extract of Citrus Peel Extract.

1.1. Synthesis of Iron Metal Complexes with Rutin

Panhwar, Q. K., & Memon, S. (2014). Methanolic solution of $\text{FeNO}_3 \cdot 9\text{H}_2\text{O}$ (1mmol) was added drop wise to a solution of Rutin (2 mmol) in methanol (10 mL) and the mixture was stirred for 2.30 h at 80°C . The mixture was cooled, and the precipitate was filtered off, washed with methanol and then air dried to obtained brown colored compounds.

1.2. Synthesis of Copper Metal Complexes with Rutin

Methanolic solution of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 10 mL (1mmol) was added drop wise to a solution of rutin (2 mmol) in methanol (10 mL) and the mixture was stirred for 15 min at

60°C . The mixture was cooled, and the precipitate was filtered off, washed with methanol and then air dried to obtain blue green colored compounds.

1.3. Synthesis of Zinc Metal Complexes with Rutin

10 mL Zinc sulphate ($\text{ZnSO}_4 \cdot 5\text{H}_2\text{O}$) (1mmol) methanolic solution was added drop wise to a solution of rutin (2 mmol) and the mixture was stirred for 20 min at 60°C . The mixture was cooled, and the precipitate was filtered off, washed with methanol and then air dried to obtain white colored compound.

1.4. Synthesis of Nickel Metal Complexes with Rutin

10 mL methanolic solution of Nickel acetate ($\text{Ni}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$) (1mmol) was added drop wise to a solution of rutin (2 mmol) in methanol (10 mL) and the mixture was stirred for 4 h at 60°C . The mixture was cooled, and the precipitate was filtered off, washed with methanol and then air dried to obtain green colored crystals.

1.5. Synthesis of Molybdenum and Zirconium Metal Complexes with Rutin

In two separate round bottom flasks, rutin (0.664 g, 2 mmol) was added to dissolve thoroughly till 15 minutes, then added zirconium nitrate (0.215 g, 1 mmol) in one flask and sodium molybdate (0.242 g, 1 mmol) in another flask under stirring containing rutin. First, the color of rutin solutions was lemon yellow; but it immediately turned deep orange after adding metal salts. After two hours of stirring at ambient temperature (Payan-Gome *et al.*, 2010). The solutions were filtered to eliminate the unreacted part of the components used; the precipitates were collected, washed with small aliquot of diethyl ether and well dried over silica gel in vacuum desiccators. The color of solid complexes was found as burnt orange and medium violet red formed with overall yield of 72% and 60% for Zr^{4+} and Mo^{+6} complexes, respectively.

2. Antioxidant Activity

Antioxidants are widely used in dietary supplements and have been investigated for the prevention of diseases such as cancer and inflammatory diseases (Barku *et al.*, 2013).

In present study, the *in vitro* antioxidant activity of citrus peels was evaluated by DPPH radical scavenging method; reducing power assay and metal chelating activity compared to the standard viz., Gallic acid, ethylene-diamine-tetraacetate (EDTA), butylated hydroxyl toluene (BHT), catechin, ascorbic acid and citric acid.

2.1. DPPH Radical Scavenging Activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl radical) scavenging activity was evaluated according to the method developed earlier and recently being followed by many researchers (Proestos *et al.*, 2013). In brief different amounts of the tested sample (50-250 µg/mL) were added to 5mL of a 0.004% methanol solution of DPPH. Finally, the absorbance was read against a blank at 517 nm after 30 min of incubation at room temperature. BHT was used as positive control while reaction mixture (DPPH radical solution) minus extract solution was taken as control.

Inhibition of free radical by DPPH in percent (IC %) was calculated by using the equation.

$$IC \% = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

Percent inhibition concentration was plotted against concentrations and the standard curve was drawn using standard antioxidant to calculate the IC₅₀ values for standard and different extracts. A lower IC₅₀ value indicated more radical scavenging activity.

2.2. Reducing Power Activity

The reducing power assay of extracts/essential oils was determined by the method developed earlier with slight modifications (Sethi *et al.*, 2015). In brief varying concentrations of tested sample were mixed with 2.5 mL of phosphate buffer (200 mM, pH= 6.6) and 2.5 mL of 1% K₄[Fe(CN)₆]. After incubation, 2.5 mL of Cl₃CCOOH was added to the mixtures, followed by centrifugation at 650 rpm for 10 min. The upper layer was mixed with 5mL of distilled water and 1 mL of 0.1% FeCl₃. Absorbance of the resultant solutions was measured at 700 nm using Thermo Scientific (Evolution 201) UV spectrophotometer. The reducing power of samples was calculated by using the following formula:

$$RP \% = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

Percent inhibition of reducing power (RP %) was plotted against concentrations and the standard curve was drawn using standard antioxidant (BHT) to calculate the RP₅₀ values for standard and different extracts/EOs. The lower RP₅₀ values indicated greater reducing power ability.

2.3. Metal Chelating Activity

The metal chelating activity of Fe²⁺ was examined by the

methods, recently being practiced and based on the principle of the Fe²⁺ chelating ability the absorbance of ferrous iron-ferrozine complex formed was measured at 562 nm (Prakash *et al.*, 2011). To execute the experiment 0.1 mL of 2mM FeCl₂.4H₂O, 0.2mL of 5mM ferrozine and 4.7 mL of methanol was added to different concentrations of tested samples. After incubation, the absorbance of test samples was measured at 562 nm. The metal chelating activity of citrus species expressed in percentage was calculated using the following formula:

$$IC \% = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

The percent of chelating ability (IC %) was plotted against concentrations and the standard curve was drawn using standard antioxidant (EDTA) to calculate the IC₅₀ values.

2.4. Superoxide Radical Scavenging Activity

The effect of super oxide radical scavenging activity was determined by the nitroblue tetrazolium reduction method (Fu *et al.*, 2010). All readings were taken in triplicate and ascorbic acid and quarcitine was used as the standard. The % inhibition was calculated by following equation.

$$IC \% = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

Where, A0 was the absorbance of control and A1 was the absorbance of ethanolic extract or standard.

3. Result and Discussion

Antioxidant activity

Antioxidants are widely used in dietary supplements and it has been investigated for the prevention of diseases such as cancer and inflammatory diseases (Barku *et al.*, 2013). In present study, the *in vitro* antioxidant activity of citrus peels was evaluated by DPPH radical scavenging method; reducing power assay and metal chelating activity compared to the standard viz., Gallic acid, ethylene-diamine-tetraacetate (EDTA), butylated hydroxyl toluene (BHT), catechin, ascorbic acid and citric acid.

DPPH radical scavenging activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl radical) scavenging activity was evaluated according to the method developed earlier and recently being followed by many researchers (Proestos *et al.*, 2013). Butylated hydroxyl toluene was used as positive control while reaction mixture (DPPH radical solution) minus extract solution was taken as control. Inhibition of free radical by DPPH in percent (IC %) was calculated by using the equation.

$$IC \% = \frac{(\text{Control} - \text{Sample})}{\text{Control}} \times 100$$

Antioxidant activity of metal complexes and bioconjugate of citrus peel extract

In present study, antioxidant activity of metal complexes of Rutin and bioconjugate of citrus peel extract was investigated using five different assays as follows.

DPPH radical scavenging activity

Mean percent DPPH radical scavenging activity of metal

complexes of Rutin and bioconjugate of citrus peel extract (De Souza and De Giovanni, 2004) in a dose depended manner with minimum at lower and maximum at higher dose level (100 µg/mL-500 µg/mL) are presented in Table 1 and Fig 2A. The radical scavenging potential of synthesized metal complexes in the form of their significant ($p < 0.05$) IC_{50} values revealed the order as Rut-Zr > Rut-Mo > Rut-Zn > Cit-Zn > Cit-Mo > Cit-Zr > Rut-Fe > Cit-Cu > Rut-Ni > Rut-Cu > Cit-Fe > Cit-Ni (Table 2 Fig 2B) IC_{50} values of standards were found in the order as Rutin ($IC_{50}=250$).

Table 1. Percent DPPH radical scavenging activity of Metal complexes of Rutin and bioconjugate of citrus peel extract collected from Uttarakhand region at different concentration

S.No.	Metal complexes	% DPPH radical scavenging activity at 700 nm.				
		100µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/mL
1	Rut-Fe	51.67±5.774	65.15±6.198	71±5.427	75.45±0.508	80.56±0.593
2	Rut-Cu	46.71±0.376	54.35±0.052	62.03±1.169	78.22±0.076	84.99±0.11
3	Rut-Ni	43.93±0.497	62.37±0.444	70.81±0.313	76±10	80.67±0.577
4	Rut-Zn	41.92±0.55	62.46±1.746	71.14±0.145	76.22±3.755	82.63±0.65
5	Rut-Zr	28.48±0.65	32.22±0.554	42.32±1.046	67.95±0.734	78.73±0.719
6	Rut-Mo	32.23±0.577	46.84±0.755	56.83±0.271	65.11±0.526	72.57±0.82
7	Cit-Fe	42.38±0	50.39±0.439	60.75±0.133	70.32±0.306	77.08±0.951
8	Cit-Cu	31±0.10	45.37±0.641	56.25±1.097	64.62±1.764	78.73±0.723
9	Cit-Ni	36.19±0.826	60±11.962	63.97±6.34	68.41±1.198	78.3±0.751
10	Cit-Zn	31.33±0.669	43.4±0.52	43.91±3.897	55.33±0.577	64.44±0.548
11	Cit-Zr	28.33±0.577	44.22±0.697	49.85±2.312	54.78±1.344	66.23±0.955
12	Cit-Mo	35.04±1.055	46.82±0.635	56.04±1.002	64.6±1.249	71.1±1.054
13	Rut*	36.33±1.528	41±10	52.33±2.517	61.9±0.475	71.48±0.289

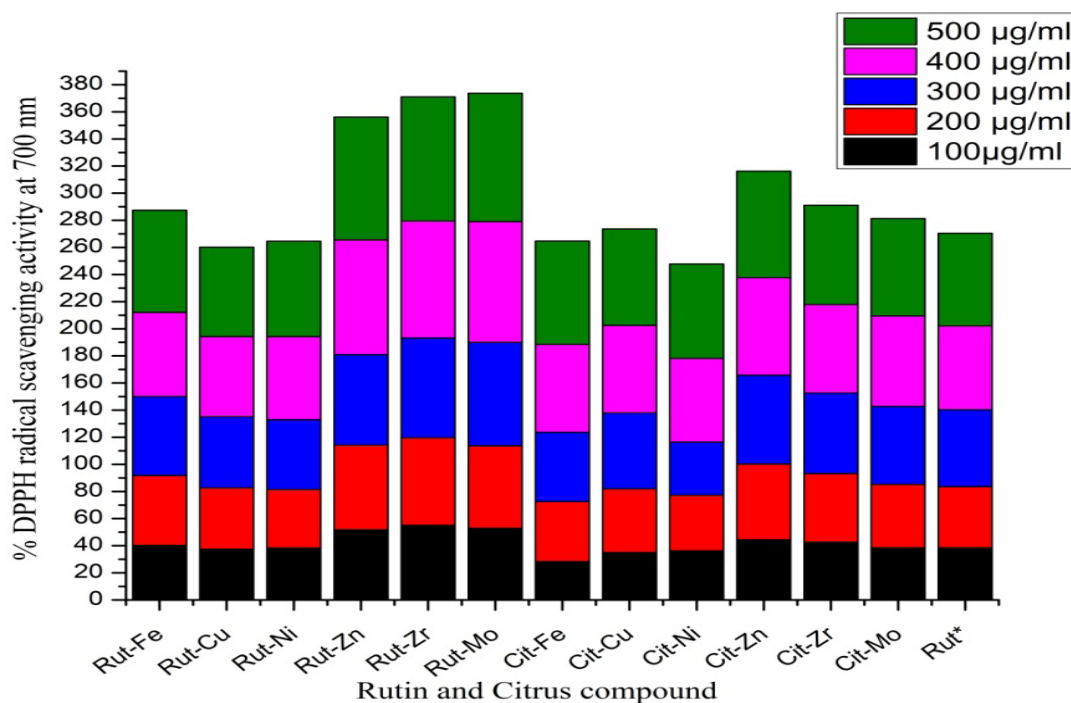
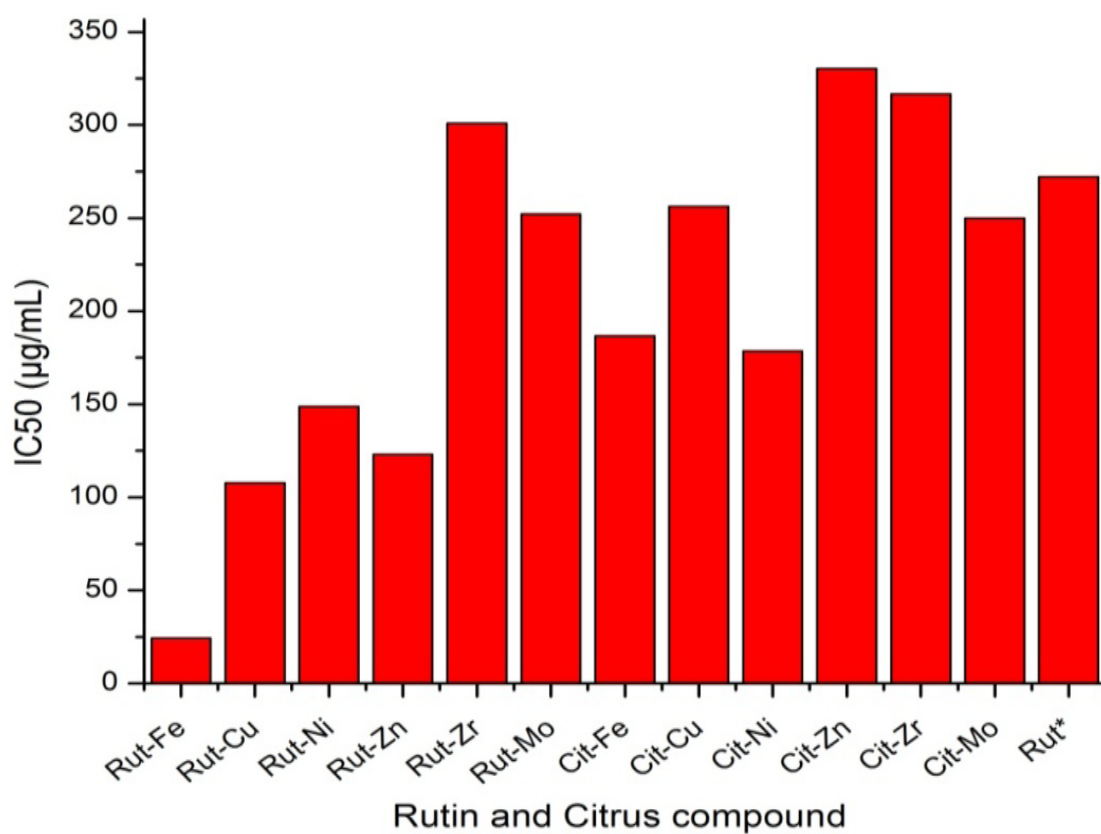


Figure 2A. Percent DPPH radical scavenging activity of metal complexes of Rutin and Bioconjugate of citrus peel extract collected from Uttarakhand region at different concentrations

Table 2. IC₅₀ of metal complexes of Rutin and bioconjugate of citrus peel extract for DPPH radical scavenging activity collected from Uttarakhand region at different concentrations

S.No.	Metal complexes	IC ₅₀ values (µg/mL) in triplicates			Mean IC ₅₀ values (µg/mL)
		I st	II nd	III rd	
1	Rut-Fe	24.41	24.34	23.22	23.99±0.668
2	Rut-Cu	107.81	108	108.11	107.97±0.152
3	Rut-Ni	148.7	149.31	148.11	148.71±0.6
4	Rut-Zn	122.94	122.98	123.32	123.08±0.209
5	Rut-Zr	300.95	300.1	300.17	300.41±0.472
6	Rut-Mo	252.22	256.33	326	278.18±41.461
7	Cit-Fe	186.74	181.23	181.4	183.12±3.133
8	Cit-Cu	256.31	257	258	257.1±0.85
9	Cit-Ni	178.47	179.99	180.11	179.52±0.914
10	Cit-Zn	330.38	330.23	330.45	330.35±0.112
11	Cit-Zr	316.62	320.45	319	318.69±1.934
12	Cit-Mo	250	250	252.11	250.7±1.218
13	Rut*	272.08	275.55	276	274.54±2.145

Values are mean of three replicates ± Standard deviation. Within column, mean values followed by the same letter are not significantly different according to Tukey's test ($p < 0.05$).


Figure 2B. IC₅₀ of DPPH activity of citrus accession collected from Uttarakhand at different concentration

Hydroxyl radical scavenging activity

Table 3 and Fig 2C Presented mean percent hydroxyl radical scavenging activity of metal complexes of Rutin and bioconjugate of citrus peel extract in a dose depended manner with minimum at lower and maximum at higher dose level (100µg/mL-500 µg/mL). IC₅₀ (p<0.05) values of

synthesized metal complexes for hydroxyl radical scavenging activity exhibited the order as Rut-Fe> Rut-Mo> Cit-Ni >Cit-Fe> Rut-Zn> Cit-Mo> Cit-Cu >Cit-Cu> Rut-Zr> Cit-Zr> Rut-Ni> Rut-Cu> Cit-Zn (Table 4 and Fig 2D) IC₅₀ values of standards were found in the order as Rutin (IC₅₀=169.72).

Table 3. Percent Hydroxyl radical scavenging activity of citrus peel extract collected from Uttarakhand region at different concentration

S.No	Metal complexes	% Hydroxyl radical scavenging activity at 560 nm.				
		100µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/mL
1	Rut-Fe	53.57±1.326	53.97±0.728	67.32±0.812	71.08±0.052	84.46±0.098
2	Rut-Cu	34±1.732	43.33±0.577	51.17±1.607	61.95±2.531	69.8±1.323
3	Rut-Ni	32.15±1.117	44.41±0.514	56.3±0.624	63.4±1.253	70.23±0.208
4	Rut-Zn	41.25±0.718	61.13±1.588	51.14±0.145	65.56±3.246	72.63±0.650
5	Rut-Zr	36.73±1.861	47.37±0.641	61.33±1.258	65.94±0.907	73.4±1.219
6	Rut-Mo	41.6±0.520	56.51±0.277	66.83±0.271	67.78±0.728	68.57±0.82
7	Cit-Fe	40.38±0	57.73±1.016	65.08±2.754	66.98±0.271	76.75±1.174
8	Cit-Cu	42.94±1.330	49.89±0.023	56.16±0.706	67.62±0.826	78.73±0.723
9	Cit-Ni	46.19±0.826	52±3.721	60.3±0.318	68.41±1.198	70.64±0.271
10	Cit-Zn	23.45±0.672	34.67±1.528	39±1	52.67±1.528	64.67±1.528
11	Cit-Zr	36.3±1.113	41.78±1.603	59.36±0.271	61.11±0.277	71.97±0.058
12	Cit-Mo	43±1	49±1	59.52±0	61.9±0.475	63.81±0.48
13	Rut*	44±1	53.37±0.548	61±1	75.1±0.458	87.67±1.528

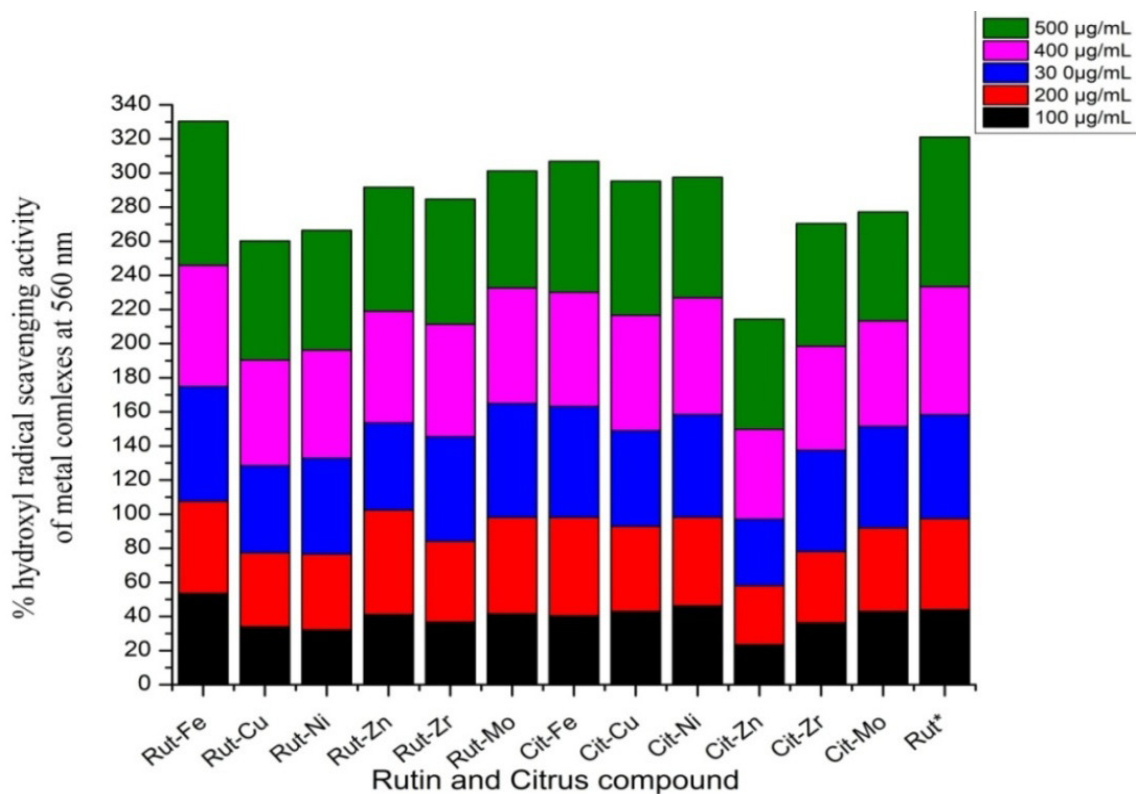
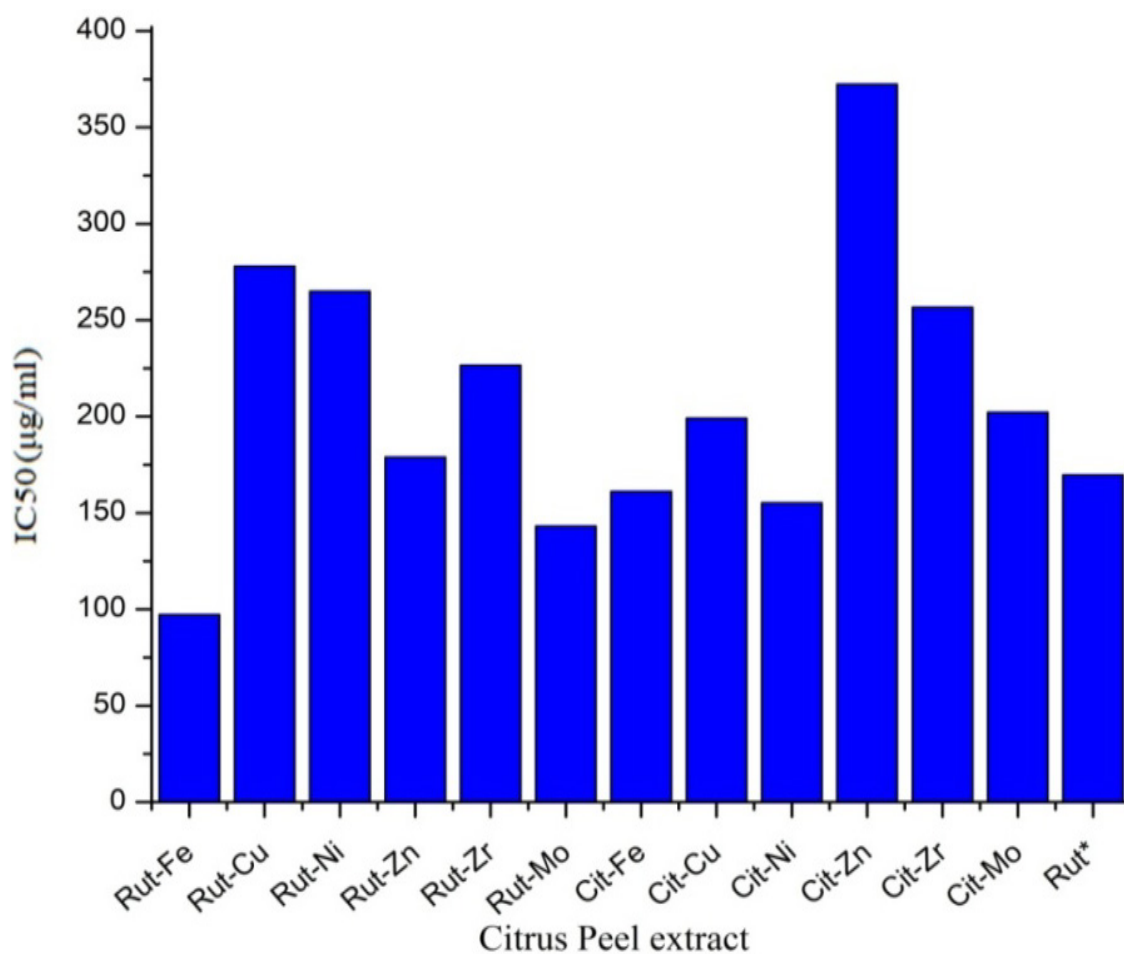


Figure 2C. Percent Hydroxyl radical scavenging activity of metal complexes of Rutin and Bioconjugate of citrus peel extract collected from Uttarakhand region at different concentrations

Table 4. IC₅₀ of metal complexes of Rutin and bioconjugate of citrus peel extract for Hydroxyl radical scavenging activity collected from Uttarakhand region at different concentrations

S.No.	Metal complexes	IC ₅₀ values (µg/mL) in triplicates			Mean IC ₅₀ values (µg/mL)
		I st	II nd	III rd	
1	Rut-Fe	97.3	97.3	96.9	97.17±0.231 ^m
2	Rut-Cu	278	277.44	276	277.15±1.032 ^b
3	Rut-Ni	265	263.5	264.5	264.33±0.764 ^c
4	Rut-Zn	179	179.78	178	178.93±0.892 ^h
5	Rut-Zr	226.59	225.55	224.5	225.55±1.045 ^e
6	Rut-Mo	143.23	144.1	143.23	143.52±0.502 ^l
7	Cit-Fe	161.21	160	161.11	160.77±0.672 ^j
8	Cit-Cu	199.21	199.21	198.34	198.92±0.502 ^g
9	Cit-Ni	155.23	155.23	155.35	155.27±0.069 ^k
10	Cit-Zn	372.4	373.341	375	373.58±1.316 ^a
11	Cit-Zr	256.66	256	255.5	256.05±0.582 ^d
12	Cit-Mo	202.3	205.92	205	204.41±1.882 ^f
13	Rut*	169.72	169.2	169.4	169.44±0.262 ⁱ

Values are mean of three replicates ± Standard deviation. Within column, mean values followed by the same letter are not significantly different according to Tukey's test (p<0.05).


Figure 2D. IC₅₀ of Metal complexes of Rutin and bioconjugate of citrus peel extract collected from Uttarakhand region for Hydroxyl radical inhibition scavenging activity

Superoxide anion scavenging activity

Mean percent superoxide anion scavenging activity of metal complexes of Rutin and bioconjugate of citrus peel extract in a dose depended manner with minimum at lower and maximum at higher dose level (100µg/mL-500 µg/mL) are presented in Table 5 and fig 2E. The superoxide anion

scavenging potential of synthesized metal complexes in the form of their significant ($p < 0.05$), IC_{50} values revealed the order as Rut-Fe > Cit-Zn > Cit-Fe > Cit-Fe > Rut-Zn > Cit-Ni > Cit-Mo > Rut-Zr > Cit-Zr > Rut-Mo > Rut-Ni > Rut-Cu Table 6 and Fig 2F the IC_{50} values of standards were found in the order as Rutin ($IC_{50}=250$).

Table 5. Percent superoxide radical anion scavenging activity of citrus peel extract collected from different region of Uttarakhand at different concentration

S.No	Metal complexes	% superoxide radical anion scavenging activity at 560 nm.				
		100µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/MI
1	Rut-Fe	41.04±1.12	50.7±0.936	67.32±0.812	71.75±1.207	84.46±0.098
2	Rut-Cu	24.19±1.612	34.52±1.775	38.45±1.018	42±1	66.33±0.577
3	Rut-Ni	25.93±0.586	38.48±0.501	44.34±1.658	55.45±0.508	70.56±0.593
4	Rut-Zn	36.71±0.376	44.35±0.052	52.03±1.169	65.22±0.076	72.65±1.045
5	Rut-Zr	33.93±0.497	42.37±0.444	50.81±0.313	56±1	75.74±1.512
6	Rut-Mo	38.41±0.271	47.19±0.287	56.76±0.287	61.74±0.271	68.38±0
7	Cit-Fe	18.48±0.65	34.89±1.755	42.32±1.046	57.95±0.734	78.4±0.252
8	Cit-Cu	42.54±0.277	50.6±0.271	59.52±0	65.24±0.745	73.24±1.076
9	Cit-Ni	42.38±0	50.39±0.439	60.75±0.133	70.32±0.306	77.08±0.951
10	Cit-Zn	31±1	45.37±0.641	56.25±1.097	64.62±1.764	78.73±0.723
11	Cit-Zr	36.19±0.826	60±11.962	63.97±6.34	68.41±1.198	78.3±0.751
12	Cit-Mo	31.33±0.669	43.4±0.52	43.91±3.897	55.33±0.577	68.78±0.029
13	Rut*	28.33±0.577	44.22±0.697	51.15±1.227	64.78±1.344	76.23±0.955

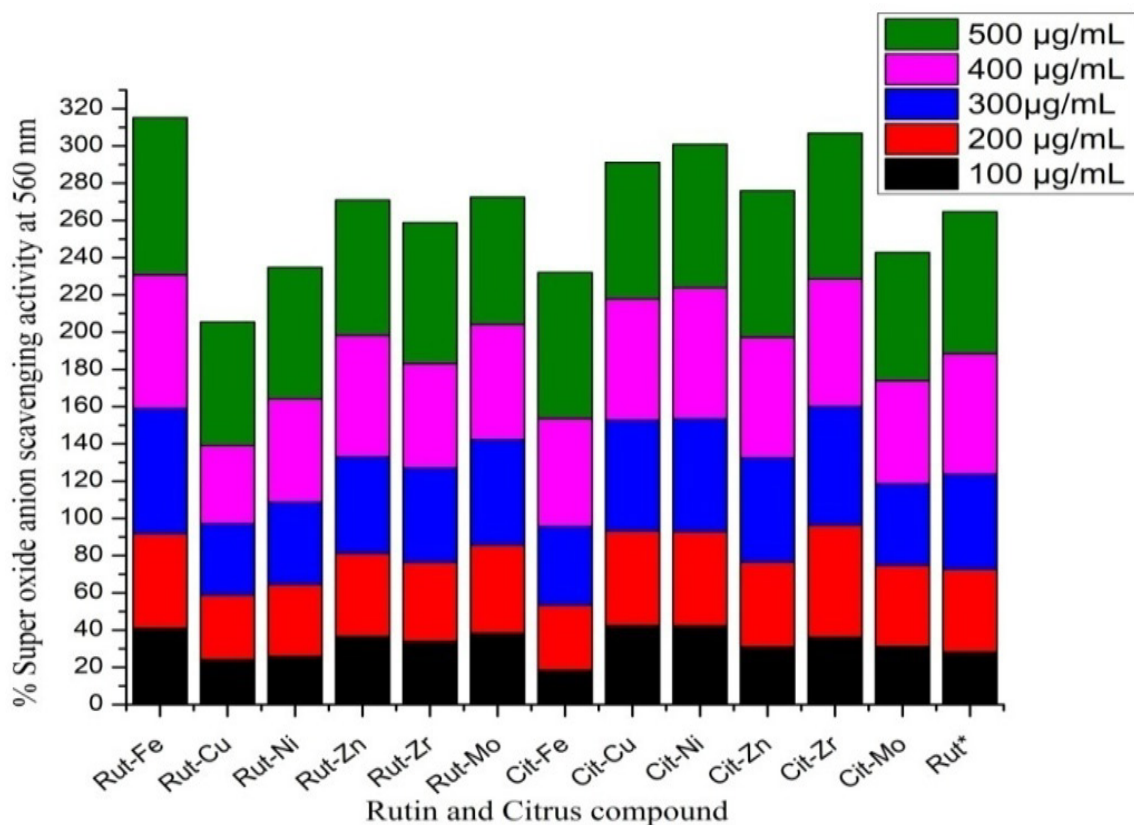
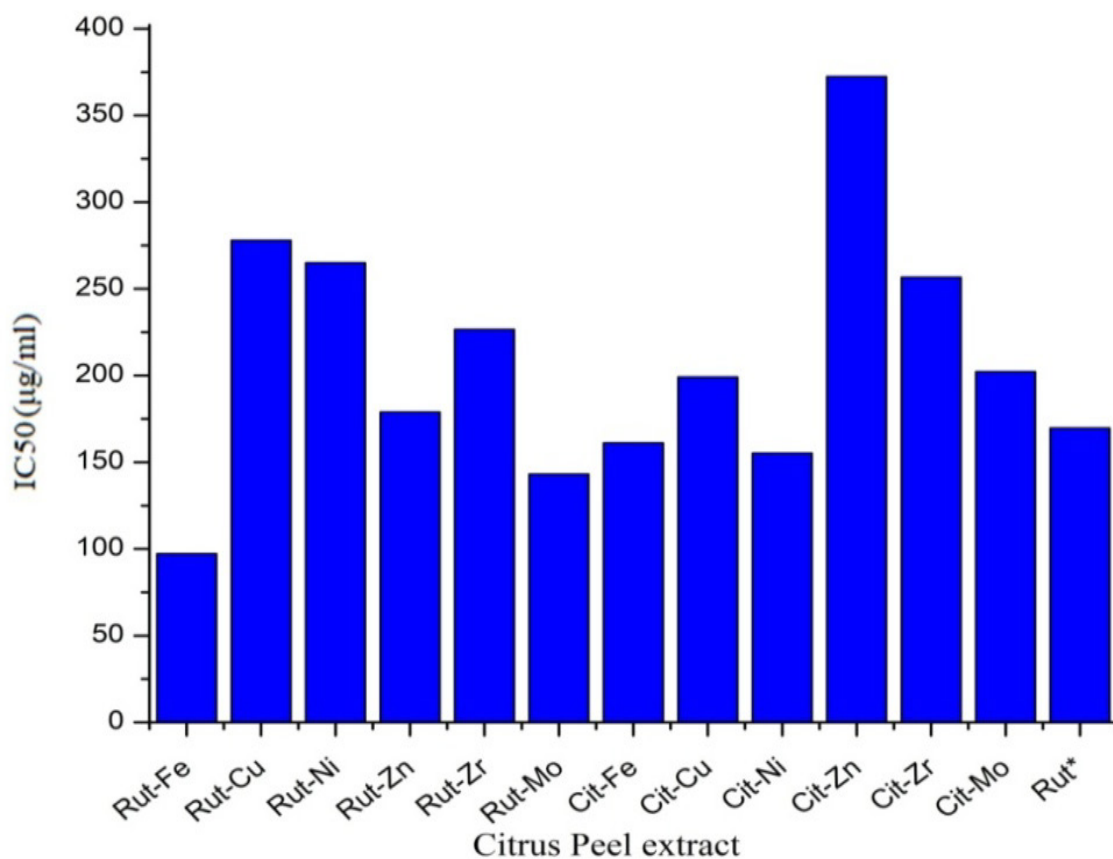


Figure 2E. Percent superoxide anion scavenging activity of metal complexes of Rutin and Bioconjugate of citrus peel extract collected from Uttarakhand region at different concentrations

Table 6. IC₅₀ of Metal complexes of Rutin and citrus peel extract for superoxide radical scavenging activity collected from Uttarakhand region at different concentrations

S.No	Metal complexes	IC ₅₀ values (µg/mL) in triplicates			Mean IC ₅₀ values (µg/mL)
		I st	II nd	III rd	
1	Rut-Fe	180	180.34	181.34	180.56±0.697
2	Rut-Cu	400.32	400	399.91	400.08±0.215
3	Rut-Ni	329.43	328.44	227	294.96±58.854
4	Rut-Zn	256.43	256.44	255	255.96±0.829
5	Rut-Zr	282.57	280	280	280.86±1.484
6	Rut-Mo	327.19	326.33	326	326.51±0.614
7	Cit-Fe	191.97	191.23	191.4	191.53±0.388
8	Cit-Cu	186.74	185	184.6	185.45±1.138
9	Cit-Ni	256.31	255	258.34	256.55±1.683
10	Cit-Zn	178.42	180.33	185.34	181.36±3.574
11	Cit-Zr	319.76	320.45	319	319.74±0.725
12	Cit-Mo	275.6	280	278.11	277.9±2.207
13	Rut*	306.17	305.55	306	305.91±0.32

Values are mean of three replicates ± Standard deviation. Within column, mean values followed by the same letter are not significantly different according to Tukey's test (p<0.05).


Figure 2F. IC₅₀ of Metal complexes of Rutin and bioconjugate of citrus peel extract collected from Uttarakhand region for Superoxide radical inhibition scavenging activity

Metal chelating activity

Table 7 and Fig 2G presented mean percent metal chelating activity of metal complexes of Rutin and bioconjugate of citrus peel extract in a dose depended manner with minimum at lower and maximum at higher dose level (100µg/mL-500 µg/mL). IC₅₀ (p<0.05) values of

synthesized metal complexes for metal chelating activity exhibited the order as Rut-Zr> Rut-Cu> Rut-Zn>Rut-Fe >Cit-Zr> Cit-Ni> Rut-Ni> Rut-Mo >Cit-Zn> Cit-Mo> Cit-Fe> Cit-Cu (Table 8 Fig 2H) IC₅₀ values of standards were found in the order as Rutin (IC₅₀=250).

Table 7. Percent Metal chelating activity of metal complexes of Rutin and Bioconjugate of citrus peel extract collected from Uttarakhand region at different concentrations

S.No.	Metal complexes	% Metal chelating activity at 560 nm.				
		100µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/mL
1	Rut-Fe	44.37±6.894	50.7±0.936	67.32±0.812	71.08±0.052	84.46±0.098
2	Rut-Cu	44.19±2.645	54.52±1.775	66.45±3.423	71±1	76.33±0.577
3	Rut-Ni	35±0	48.48±0.501	54.34±1.658	65.45±0.508	70.56±0.593
4	Rut-Zn	46.71±0.376	54.35±0.052	62.03±1.169	78.22±0.076	84.99±0.11
5	Rut-Zr	43.93±0.497	62.37±0.444	70.81±0.313	76±1	80.67±0.577
6	Rut-Mo	31.92±0.55	43.13±1.038	51.14±0.145	68.22±0.4	72.63±0.65
7	Cit-Fe	28.48±0.65	34.89±1.755	42.32±1.046	57.95±0.734	68.73±0.719
8	Cit-Cu	22.23±0.577	36.51±0.277	46.83±0.271	55.11±0.526	62.57±0.82
9	Cit-Ni	42.38±0	50.39±0.439	60.75±0.133	70.32±0.306	77.08±0.951
10	Cit-Zn	31±1.0	45.37±0.641	56.25±1.097	64.62±1.764	78.73±0.723
11	Cit-Zr	36.19±0.826	60±11.962	63.97±6.34	68.41±1.198	78.3±0.751
12	Cit-Mo	31.33±0.669	43.4±0.52	43.91±3.897	55.33±0.577	64.44±0.548
13	Rut*	28.33±0.577	44.22±0.697	51.15±1.227	64.78±1.344	76.23±0.955

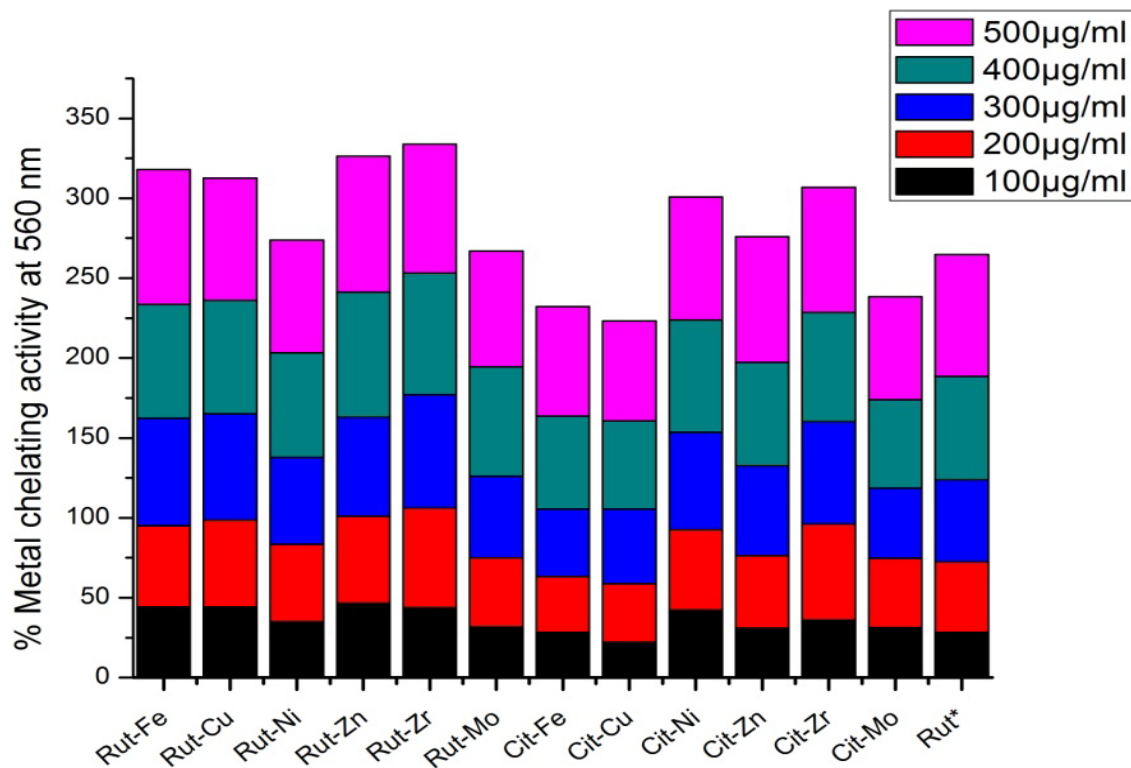
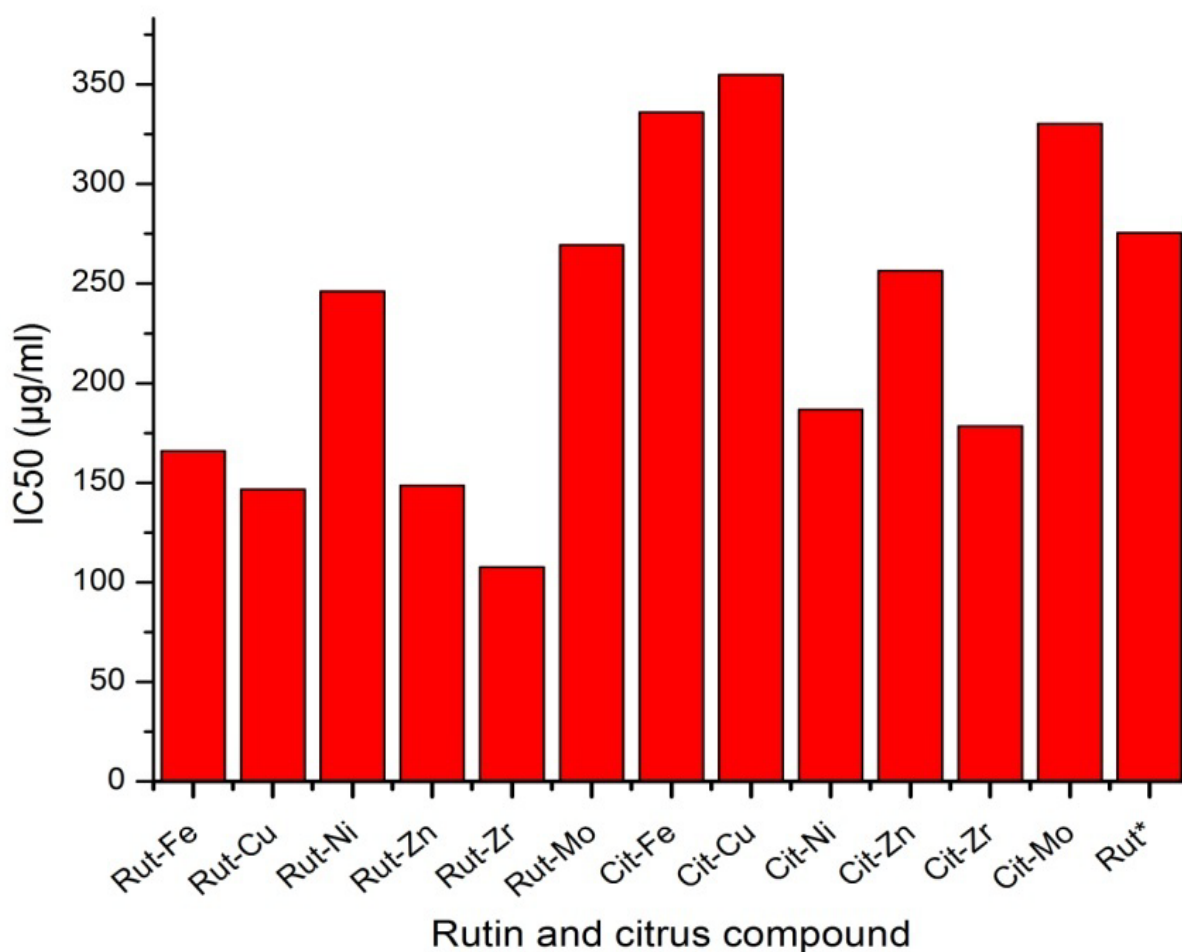


Figure 2G. Percent Metal chelating activity of metal complexes of Rutin and Bioconjugate of citrus peel extract collected from Uttarakhand region at different concentrations

Table 8. IC₅₀ of Metal complexes of rutin and bioconjugate of citrus peel extract for Metal chelating activity collected from Uttarakhand region at different concentrations

S.No	Metal complex	Mean IC ₅₀ values (µg/mL) in triplicates			Mean IC ₅₀ values (µg/mL)
		I st	II nd	III rd	
1	Rut-Fe	165.9	164.34	163.22	164.49±1.346
2	Rut-Cu	146.625	148	148.11	147.58±0.827
3	Rut-Ni	246.25	249.31	248.11	247.89±1.542
4	Rut-Zn	148.7	148.98	145.32	147.67±2.037
5	Rut-Zr	107.81	100.1	105.17	104.36±3.918
6	Rut-Mo	269.33	266.33	266	267.22±1.835
7	Cit-Fe	335.92	335.23	333.4	334.85±1.302
8	Cit-Cu	354.94	257	258	289.98±56.259
9	Cit-Ni	186.74	179.99	180.11	182.28±3.863
10	Cit-Zn	256.31	250.23	250.45	252.33±3.449
11	Cit-Zr	178.47	180.45	180	179.64±1.038
12	Cit-Mo	330.38	330	332.11	330.83±1.125
13	Rut*	275.6	275.55	276	275.72±0.247

Values are mean of three replicates ± Standard deviation. Within column, mean values followed by the same letter are not significantly different according to Tukey's test (p<0.05).


Figure 2H. IC₅₀ Metal complexes of Rutin and bioconjugate of citrus peel extract collected from Uttarakhand region for Metal chelating activity

Reducing power activity

Mean percent reducing power activity of metal complexes of Rutin and bioconjugate of citrus peel extract in a dose depended manner with minimum at lower and maximum at higher dose level (100µg/mL-500 µg/mL) are presented in Table9 and Fig 2 I. The radical scavenging

potential of synthesized metal complexes in the form of their significant ($p < 0.05$) IC_{50} values revealed the order as $Cit-Fe > Rut-Cu > Cit-Zr > Cit-Ni > Cit-Cu > Cit-Zn > Rut-Zr > Cit-Mo > Rut-Fe > Rut-Mo > Rut-Zn > Rut-Ni$ (Table 10 Fig 2J) IC_{50} values of standards were found in the order as Rutin ($IC_{50}=109.65$).

Table 9. Percent reducing power activity of metal complexes of Rutin and Bioconjugate of citrus peel extract collected from Uttarakhand region at different concentrations

S.No	Metal complexes	% Reducing power activity at 700 nm				
		100µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/mL
1	Rut-Fe	45.97±0.32	40.03±0.34	32.10±0.12	35.15±1.21	25.52±0.13
2	Rut-Cu	68.13±0.11	64.21±0.33	52.05±0.77	45.36±1.11	23.44±0.11
3	Rut-Ni	66.02±0.40	62.09±0.35	50.03±0.09	45.65±0.2	25.43±0.34
4	Rut-Zn	68.23±0.51	50.45±0.22	30.02±0.08	21.28±0.19	18.20±3.04
5	Rut-Zr	65.21±0.81	61.21±0.21	51.54±0.03	39.32±0.06	25.20±1.20
6	Rut-Mo	67.02±0.22	60.03±0.22	45.32±0.04	37.04±0.12	25.40±0.06
7	Cit-Fe	58.06±0.03	40.45±0.34	36.26±0.07	28.24±0.29	23.40±1.23
8	Cit-Cu	62.03±0.07	31.00±0.45	25.20±0.23	22.22±1.33	20.04±2.11
9	Cit-Ni	53.1±0.06	48.02±0.67	43.12±0.01	34.14±2.33	20.10±0.67
10	Cit-Zn	43.1±0.44	37.08±0.22	31.09±0.01	23.14±0.13	18.07±0.99
11	Cit-Zr	58.1±0.11	52.04±0.26	45.06±0.03	32.08±0.12	20.09±0.34
12	Cit-Mo	45.34±0.12	41.26±0.22	30.28±0.04	25.28±0.11	20.0±0.22
13	Rut*	44.2±0.23	40.20±0.11	30.20±0.37	25.24±2.65	19.26±0.09

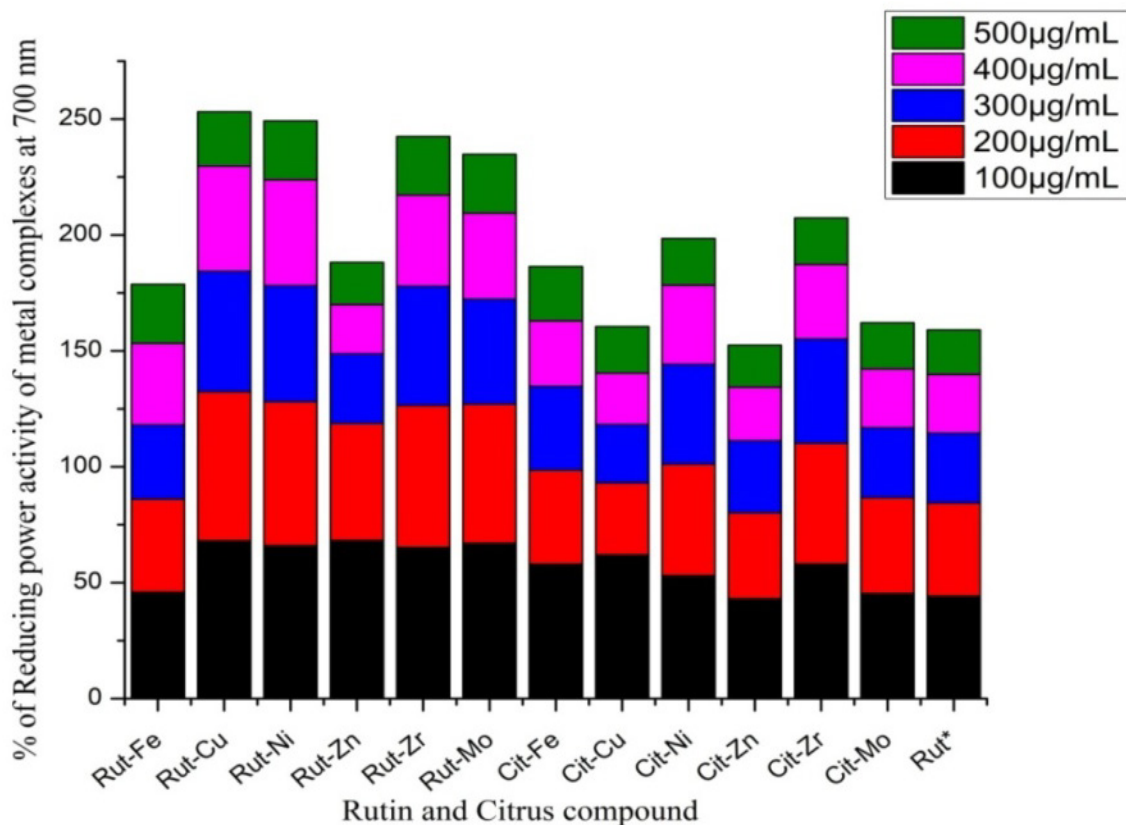
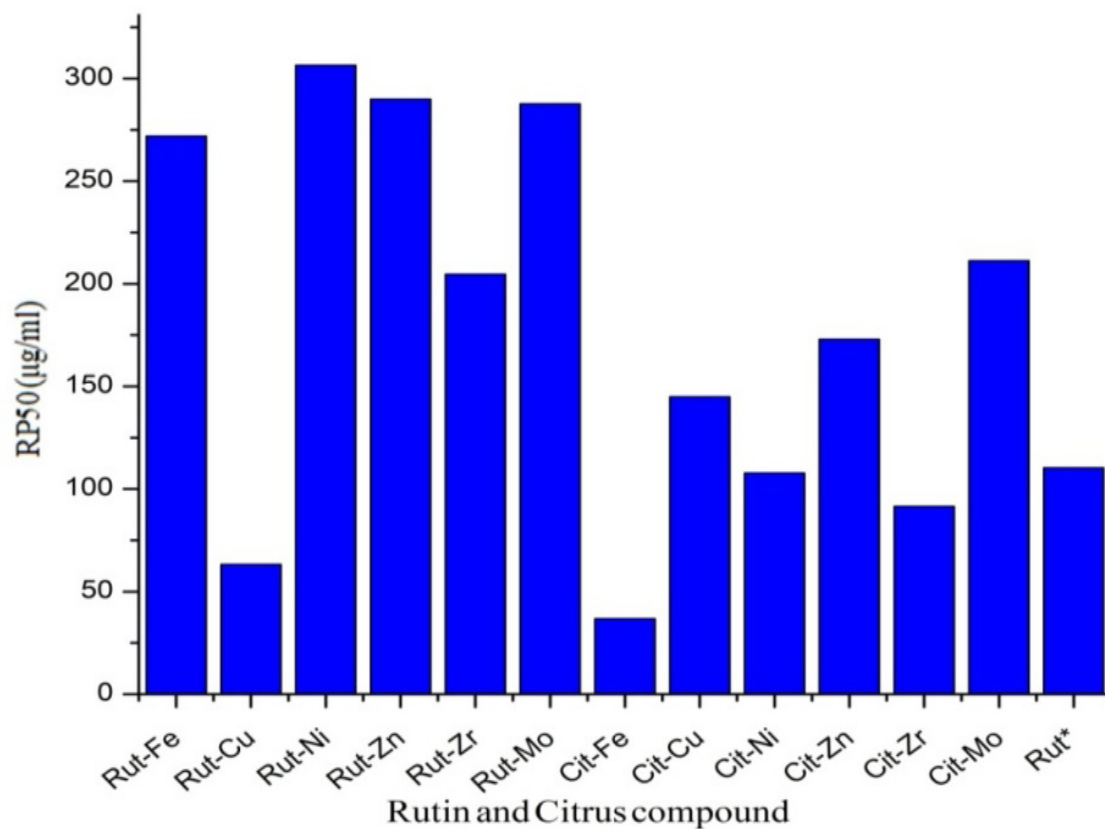


Figure 2I. Percent reducing power activity of metal complexes of Rutin and Bioconjugate of citrus peel extract collected from Uttarakhand region at different concentrations

Table 10. IC₅₀ of Metal complexes of Rutin and bioconjugate of citrus peel extract for reducing power activity collected from Uttarakhand region at different concentrations

S.No	Metal complexes	RP ₅₀ values (µg/mL) in triplicates			Mean RP ₅₀ values (µg/mL)
		I st	II nd	III rd	
1	Rut-Fe	271.98	271	272.5	271.83±0.762 ^d
2	Rut-Cu	63.27	63.27	62.08	62.87±0.687 ^l
3	Rut-Ni	306.48	306	305.5	305.99±0.49 ^a
4	Rut-Zn	290	290	289.8	289.93±0.115 ^b
5	Rut-Zr	204.65	204	204.6	204.42±0.362 ^f
6	Rut-Mo	287.72	287.72	287.5	287.65±0.127 ^c
7	Cit-Fe	36.81	36.81	34	35.87±1.622 ^m
8	Cit-Cu	144.93	144	145.5	144.81±0.757 ^h
ss9	Cit-Ni	107.82	107.82	105	106.88±1.628 ^j
10	Cit-Zn	172.91	172.95	175.6	173.82±1.542 ^e
11	Cit-Zr	91.54	92.5	92.8	92.28±0.658 ^k
12	Cit-Mo	211.25	210.92	210	210.72±0.648 ^c
13	Rut*	110.35	109.2	109.4	109.650.614 ⁱ

Values are mean of three replicates ± Standard deviation. Within column, mean values followed by the same letter are not significantly different according to Tukey's test (p<0.05).


Figure 2J. IC₅₀ of Metal complexes of Rutin and bioconjugate of citrus peel extract collected from Uttarakhand region for reducing power activity

Synthesis and characterization of metal complexes of Rutin and bioconjugate of citrus peel extract

Table 11. UV-visible Spectroscopy Analysis of metal complexes of Rutin

S.No	Complexes	I-Band (nm)	II -Band (nm)
1	Rutin	300	500
2	Rut-Fe	220	430
3	Rut-Cu	250	400
4	Rut-Mo	240	410
5	Rut-Zr	260	420
6	Rut-Ni	220	410
7	Rut-Zn	300	480

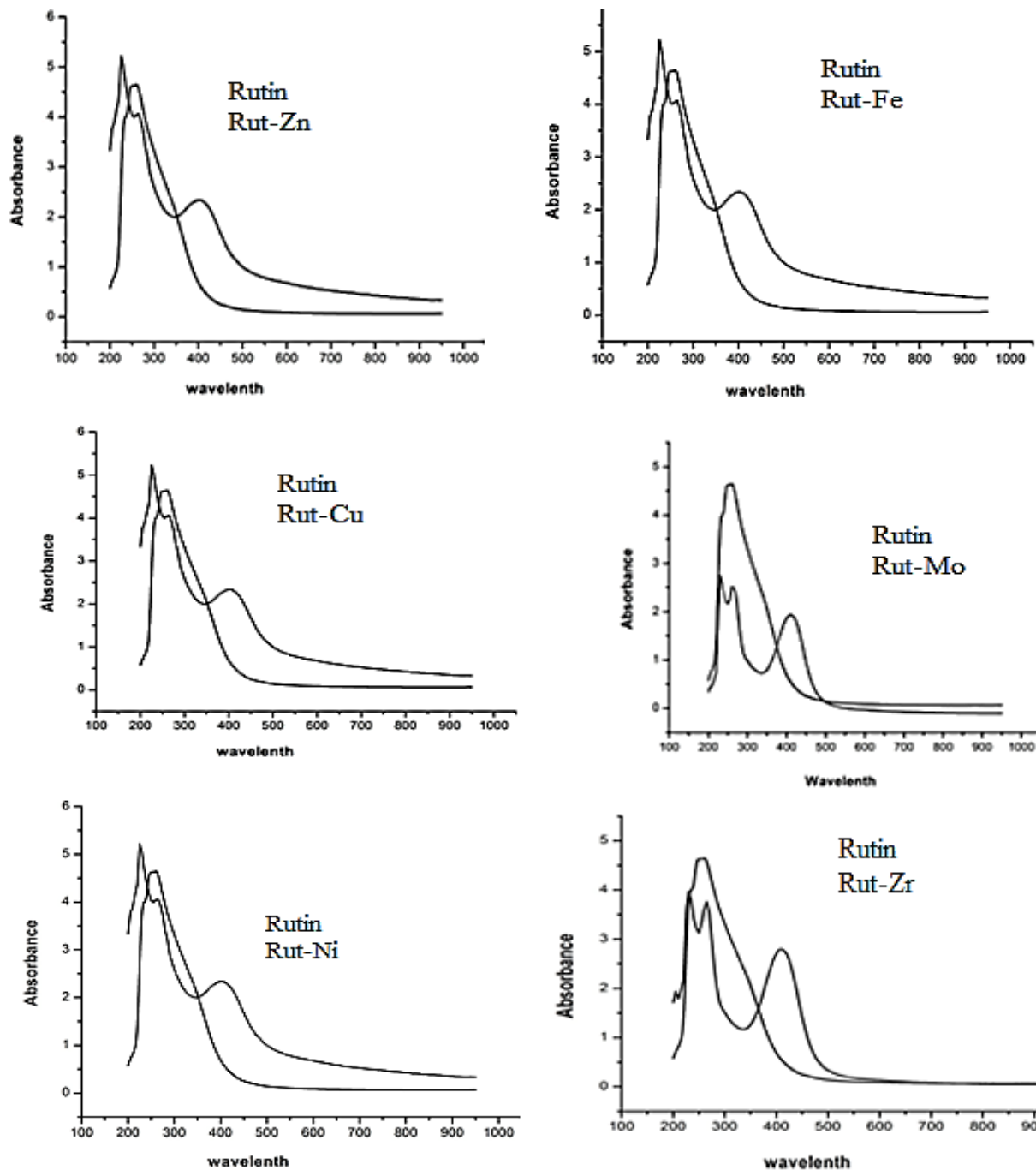


Figure 3A. UV- visible spectra of metal complexes of Rutin

Table 12. UV-visible Spectroscopy Analysis of bioconjugate of citrus extract

S.No	Complexes	I -Band (nm)	II -Band (nm)
1	Citrus	340	420
2	Citrus -Fe	300-350	450
3	Citrus -Cu	200-240	400
4	Citrus -Mo	210-240	440
5	Citrus -Zr	300	350
6	Citrus -Ni	300-340	480
7	Citrus-Zn	240-280	450

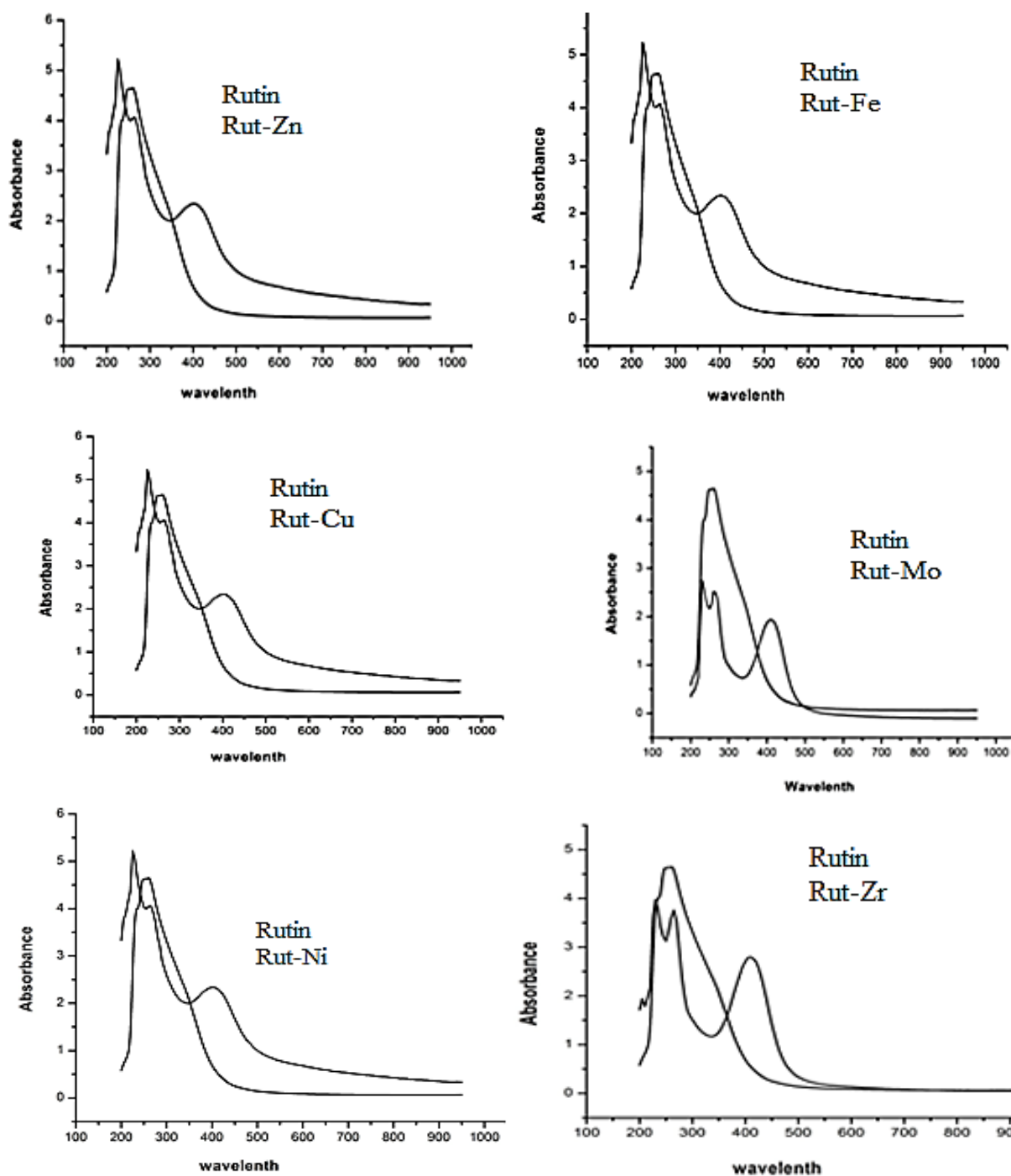


Figure 3B. UV- visible spectra of metal complexes of Rutin

Rutin has ability to effectively chelate the metal ions *via* o-dihydroxyl and 5OH-4CO groups, but involvement of particular site in chelate formation depends more on the nature of a metal ion and some other factors such as location of hydroxyl groups and steric hindrance at legating groups to affect the complexation it has been noticed that the changes in the visible spectrum of rutin (350-500 nm) depend more on the nature of metal salt, complex present and other factors. In general, the spectral changes in the 250-270 nm range are insignificant whereas, the changes in the visible range (350-500 nm) are highly significant for complexation purpose (N. B. Mel'nikova *et al.*, 2002). Similar to that, electronic spectra of rutin and its complexes show two clear visible peaks. The absorbance peaks in the visible region at 359, 422 and 413 nm are produced whilst those visualized at lower region. Around 257, 275 and 271 nm is associated to a ring (*i.e.* benzoyl system) for rutin and its UV-Vis absorption spectra of ligand Rutin and their corresponding different metal complexes (Fe, Cu, Mo, Ni, Zr and Zn) of Rutin were recorded in the range of 300 to 500 nm in DMSO (Table 11).

The shifting of bands between the ligand and metal complexes of ligand and the transition metals. Appearance of new absorption bands gives an indication of the formation of the complexes. The UV-visible spectrum of Rutin showed an instance absorbance band at 300 nm and transition between 300 to 500 nm found to be maximum at 320 nm. The UV-visible spectrum of the zinc complex shows a broad absorption band at 420 nm attributed to the bathochromic shift and the lower wavelength exhibited to the hypsochromic shift. The Iron complex shows a broad absorption band at 400 nm attributed to the bathochromic shift and the lower wavelength 200 nm exhibited to the hypsochromic shift. In Copper complex the hypsochromic shift 250 nm while absorption at 380 to 420 nm exhibited shoulder like bathochromic shift. In the UV spectrum of

Mo and Zr The spectra show the bathochromic shift of about 410 and 430 nm respectively for Mo^{+6} , Zr^{4+} and Such spectral shifts are either analogous to $\pi-\pi^*$ electronic transitions or witness the presence of charge transfer complexes (CTC) produced through the formation of coordination bonds between unshared electron pairs of oxygen atoms of the phenolic groups at rutin and the d-orbital's of molybdenum and zirconium ions.

UV-Vis absorption spectra of bioconjugate of citrus peel extract were recorded in the range of 300 to 550 nm in DMSO corresponding to metal (Fe, Cu, Mo, Ni, Zr and Zn) in Table 12. The shifting of bands between the ligand and metal complexes of ligand and the transition metals. Appearance of new absorption bands gives indications of the formation of the complexes. The UV-visible spectrum of citrus peel extract showed broad absorbance band at 310 nm and transition between 300 to 500 nm found to be maximum at 320 nm. The UV-visible spectrum of the zinc complex shows a broad absorption band at 420 nm attributed to the bathochromic shift and the lower wavelength exhibited to the hypsochromic shift. The Iron complex shows a broad absorption band at 380 nm attributed to the bathochromic shift and the lower wavelength 300 nm exhibited to the hypsochromic shift. In Copper complex the hypsochromic shift 250 nm while absorption at 380 to 420 nm exhibited shoulder like bathochromic shift. In the UV spectrum of Mo and Zr The spectra show the bathochromic shift of about 400 and 320 nm respectively for Mo^{+6} , Zr^{4+} and such spectral shifts are either analogous to $\pi-\pi^*$ electronic transitions or witness the presence of charge transfer complexes (CTC) produced through the formation of coordination bonds between unshared electron pairs of oxygen atoms of the phenolic groups at rutin and the d-orbital's of molybdenum and zirconium ions.

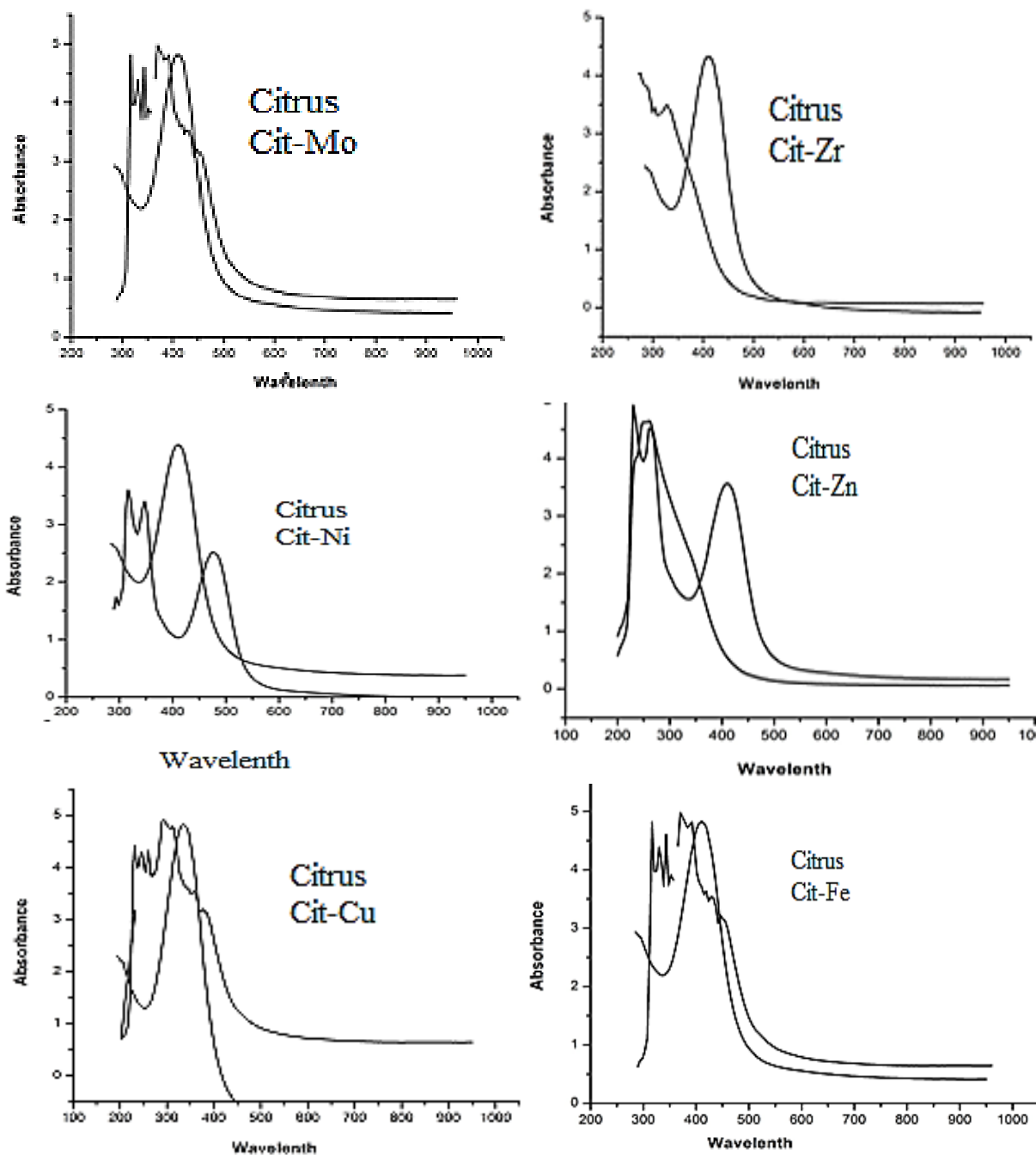


Figure 3C. UV-visible spectra of metal complexes of bioconjugate of citrus peel extract

Optical properties of metal complexes

During absorption process, transition of electron takes place from a lower to higher energy state by absorbing a photon of definite energy, depends upon the transmitted radiation. The fundamental absorption shows a sudden rise in the absorption, known as absorption edge, which can be

used to determine the optical band gap (E_g) is calculated from the relation (Tauc,s plot) from the following equation:

$$ah\nu = A (h\nu - E_g)^n$$

Where n is equal to $1/2$ and 2 for direct and indirect transition respectively.

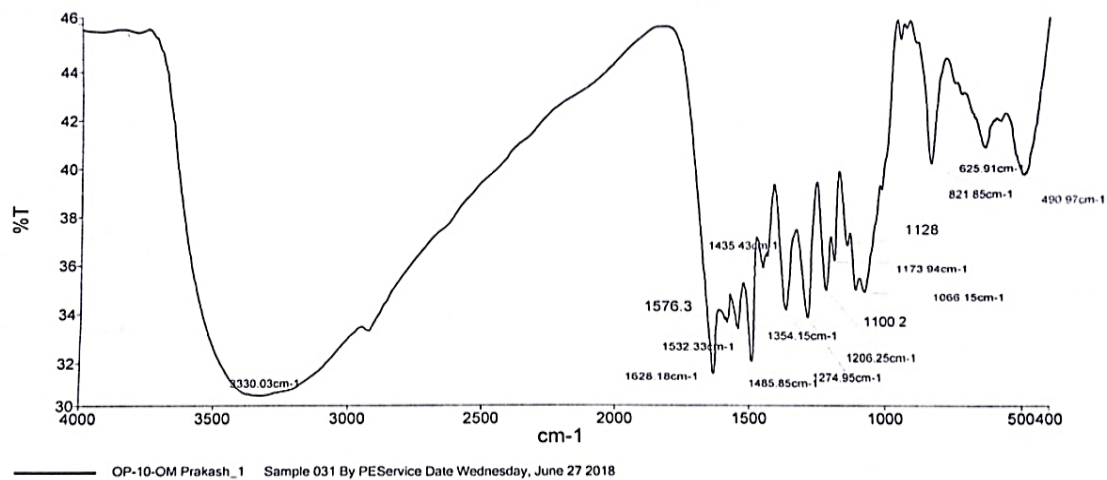


Figure 4A. FT-IR bioconjugate of Cit-Fe

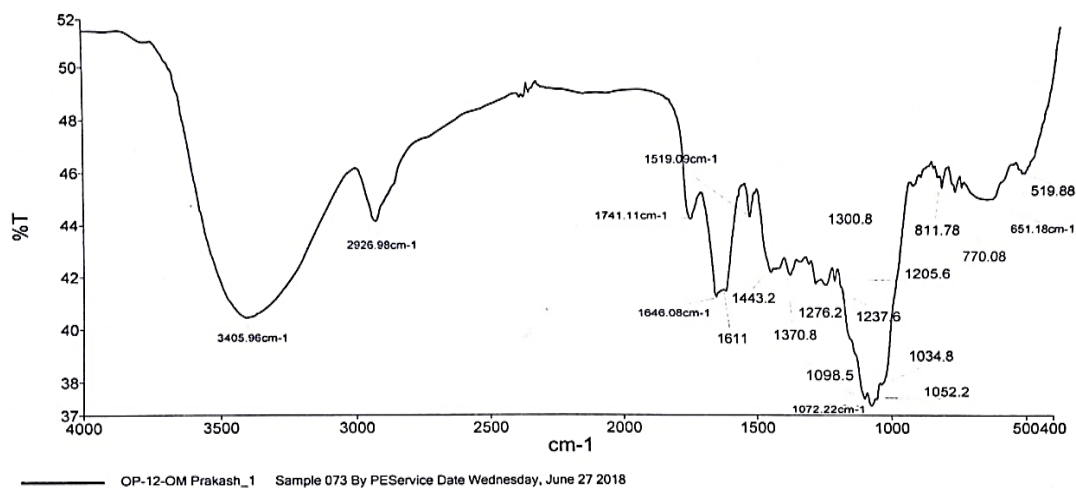


Figure 4B. FT-IR of bioconjugate Cit-Mo

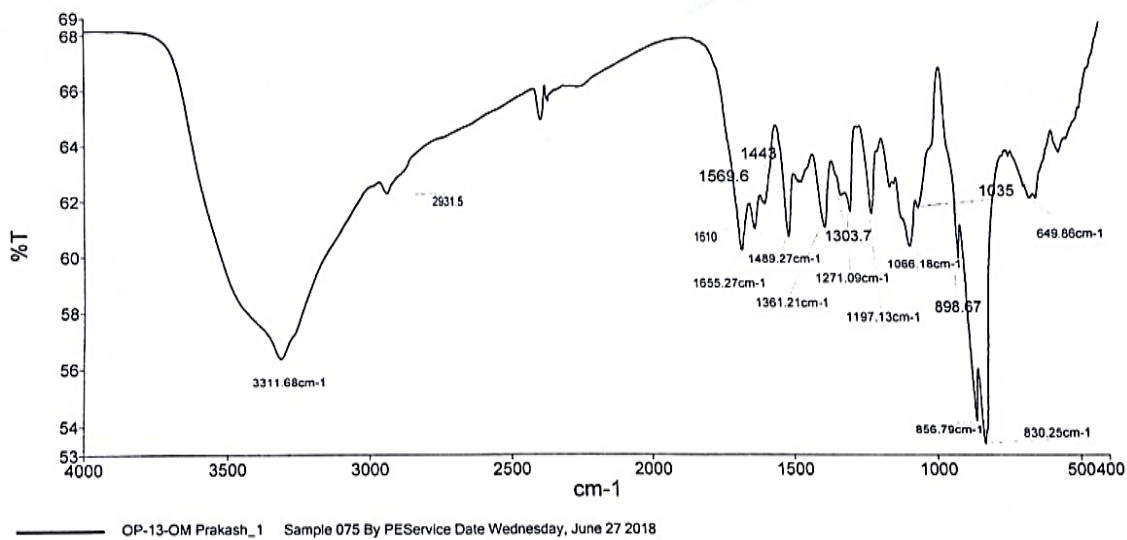


Figure 4C. FT-IR of bioconjugate of Cit-Ni

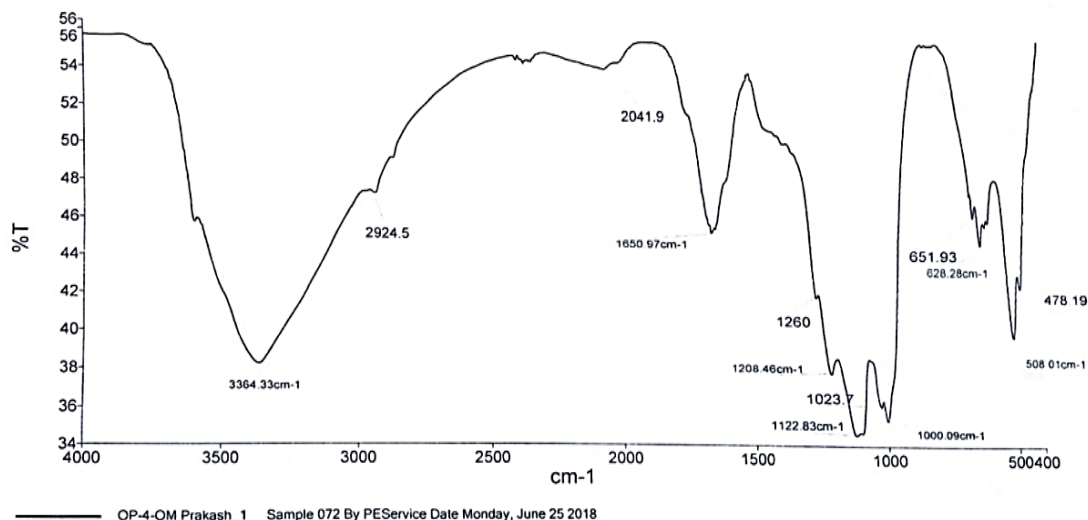


Figure 4D. FT-IR of Metal complex of Rut-Zn

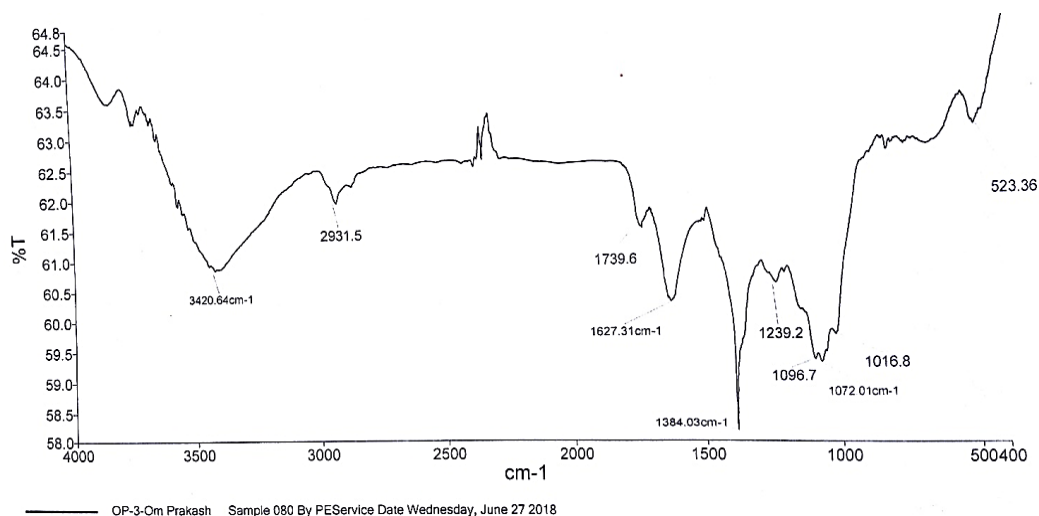


Figure 4E. FT-IR of metal complex Rut-Ni

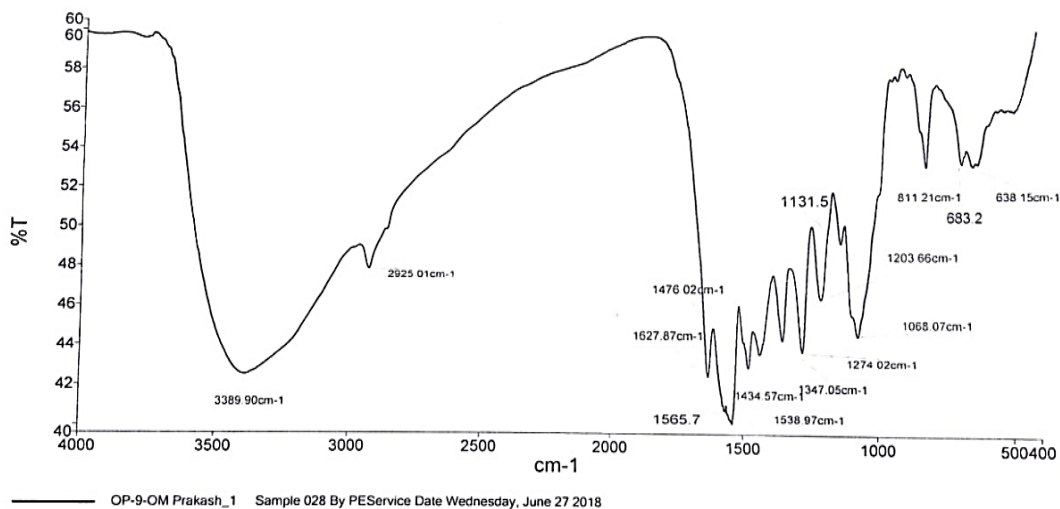


Figure 4F. FT-IR of bioconjugate of Cit-Zn

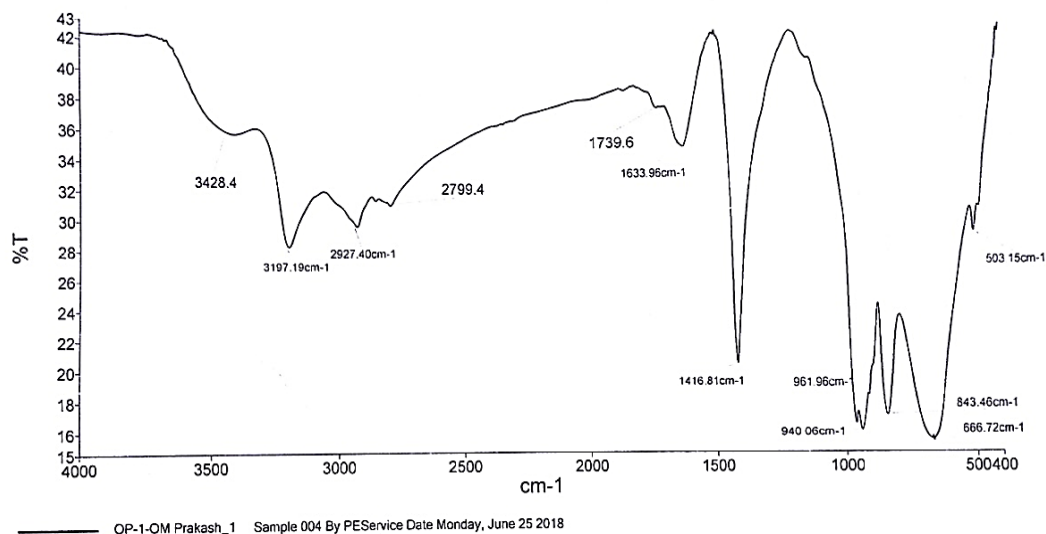


Figure 4G. FT-IR of metal complex Rut-Cu

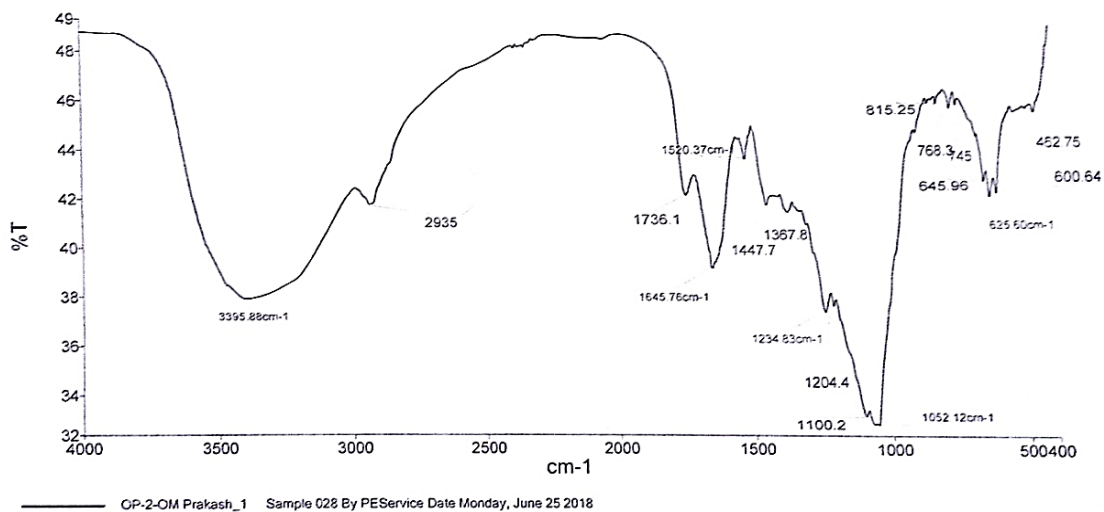


Figure 4H. FT-IR of metal complex Rut-Fe

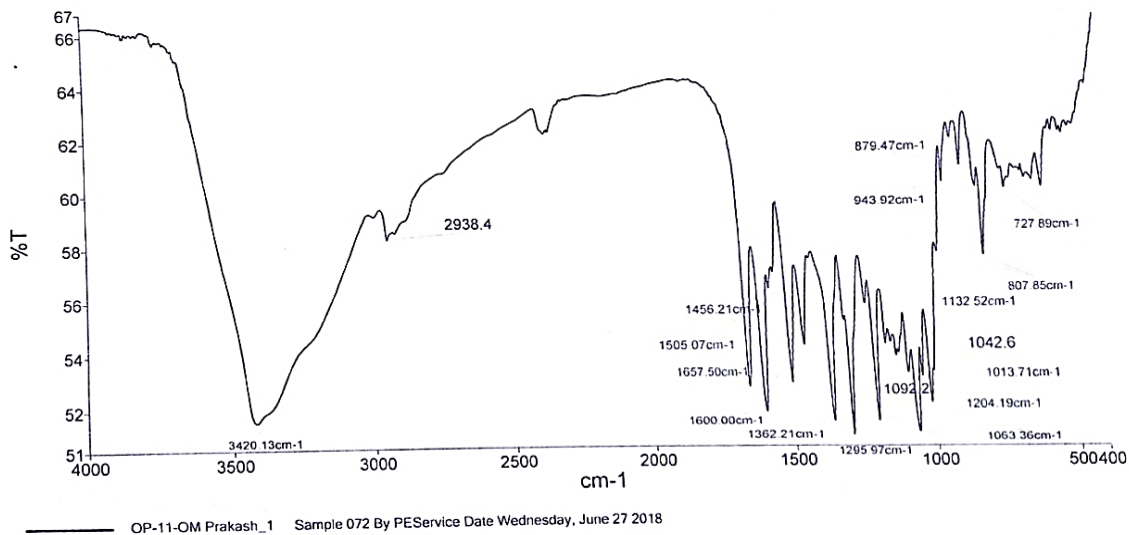
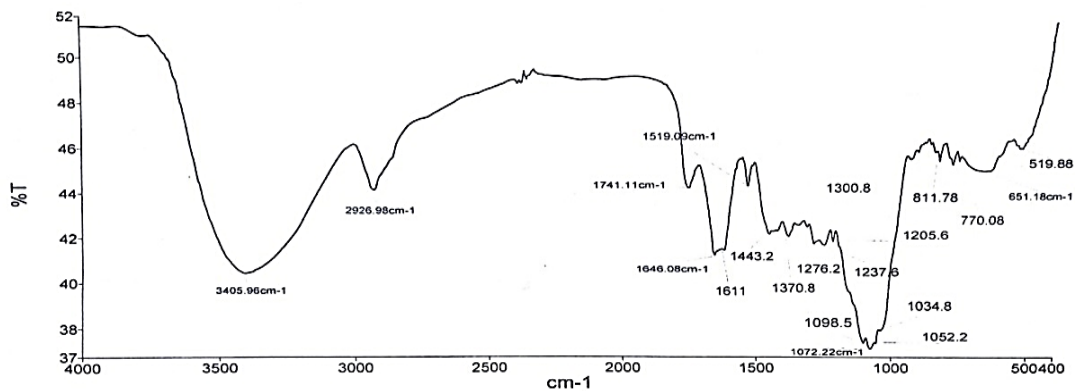
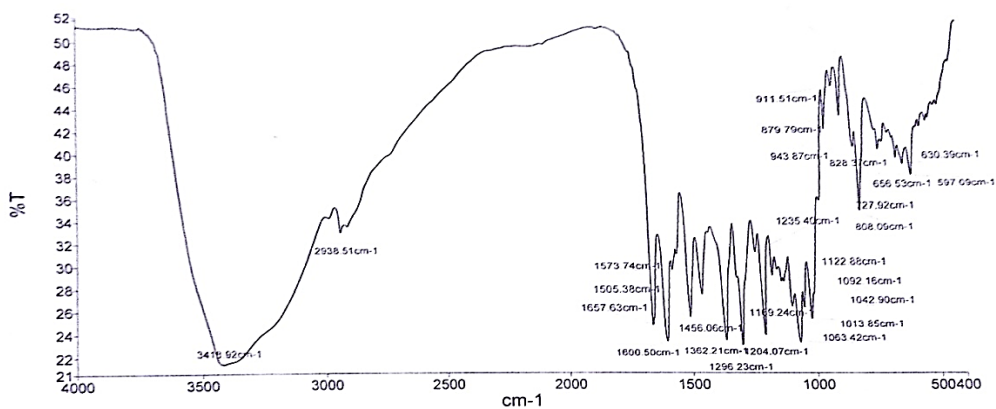


Figure 4I. FT-IR metal complex of Cit-Cu



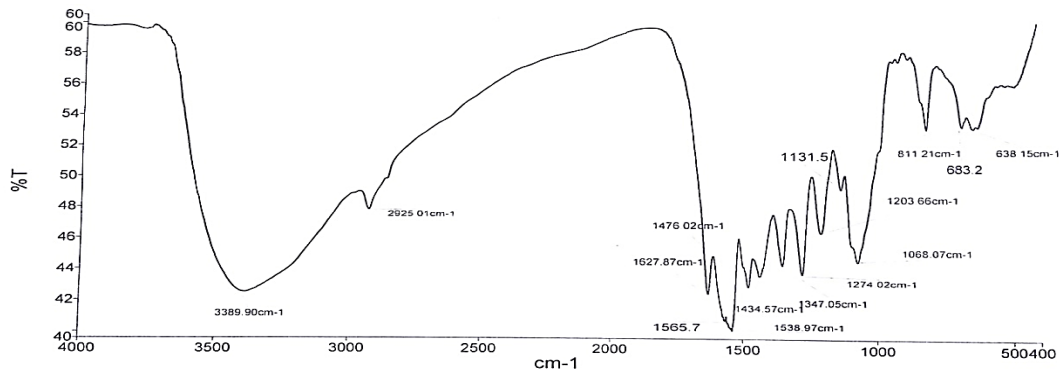
OP-12-OM Prakash_1 Sample 073 By PEService Date Wednesday, June 27 2018

Figure 4J. FT-IR of metal complex Rut-Mo



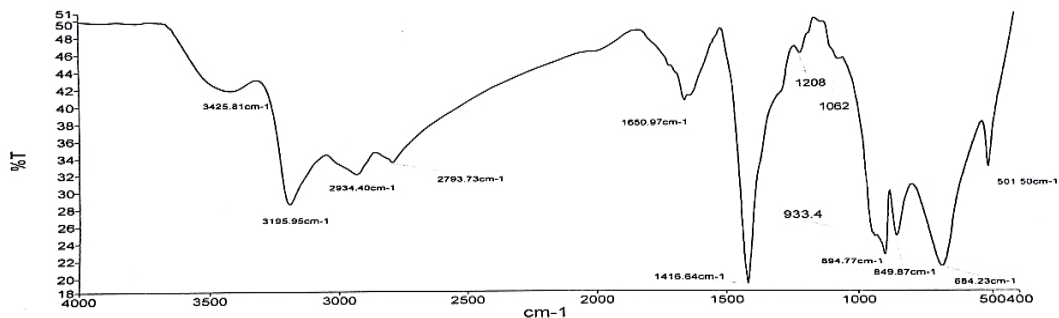
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Figure 4K. FT-IR of Rutin



OP-9-OM Prakash_1 Sample 028 By PEService Date Wednesday, June 27 2018

Figure 4L. FT-IR of Citrus



OP-8-OM Prakash_1 Sample 031 By PEService Date Monday, June 25 2018

Figure 4M. FT-IR of metal complex of Rut-Zr

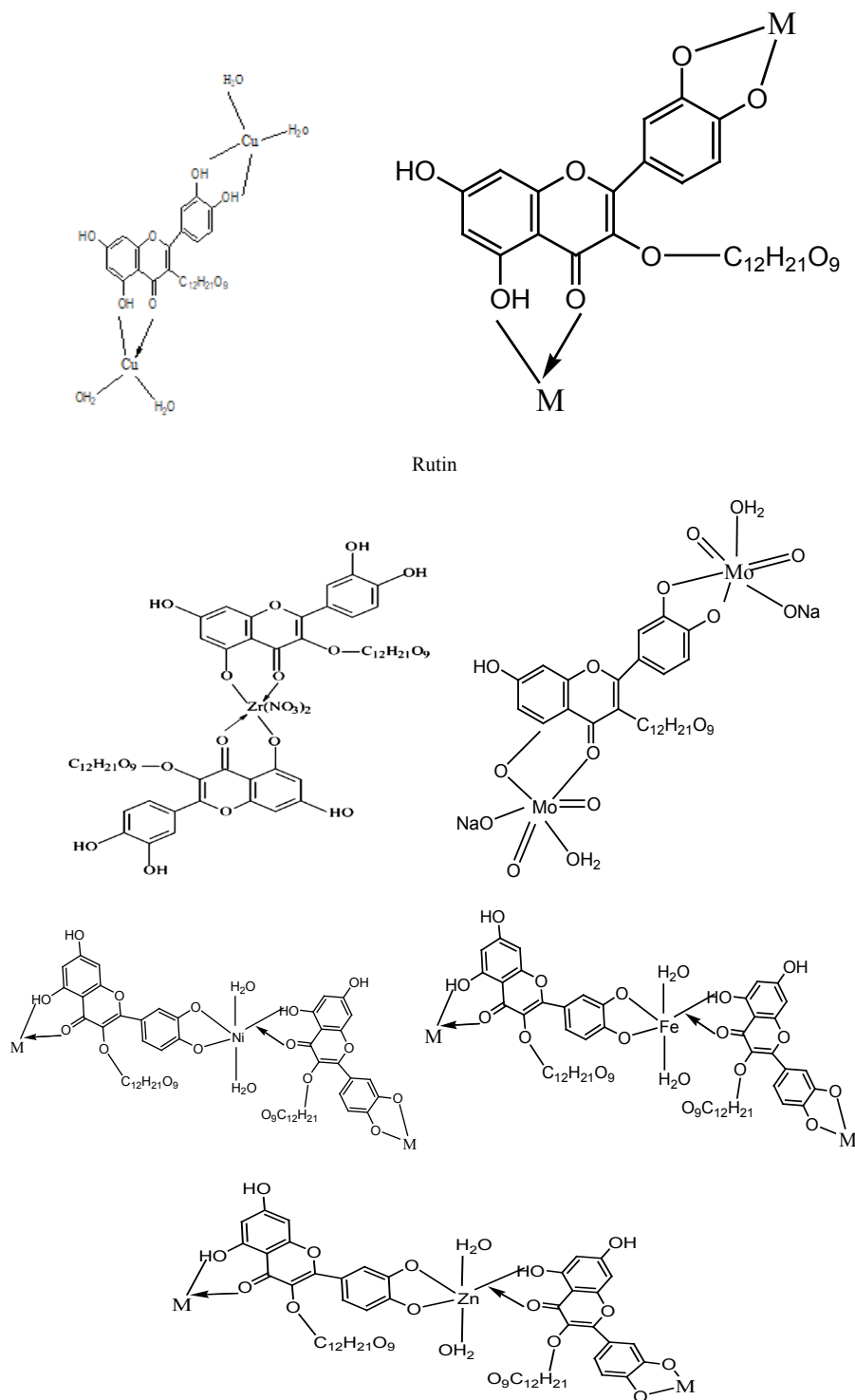


Figure 5. Metal complexes of Rutin with (Cu, Fe, Zn, Ni, Mo and Zr)

To systematically study the effects of the number of d electrons of the first transition metal ions (Fe, Co, Ni, Cu, Zr, Mo and Zn) on the formation and stability of metal flavonoid complexes of Rutin/ M^{2+} , M^{4+} , M^{6+} complex as a model system to investigate the structures and properties of these complexes. The FT-IR spectrum (Fig 4A-4M) of ligand Rutin shows characteristic absorption bands at 3410 cm^{-1} corresponding to hydroxyl stretching

$\nu(\text{O-H})$, $(\text{C-H})\text{sp}^3$ stretching, $\nu(\text{C-H})\text{sp}^2$ 2938 cm^{-1} stretching, carbonyl stretching 3418 cm^{-1} , $\nu(\text{C=C})$ aromatic ring stretching 1610 cm^{-1} , $\nu(\text{C-C})$ 1100 cm^{-1} stretching, $\nu(\text{C-O})$ 1300 cm^{-1} phenolics, $\nu(\text{OCH}_3)$ 1130 cm^{-1} stretching respectively. Band at 3410 cm^{-1} of phenolic $\nu(\text{O-H})$ stretching shifted broad peak in Nickel, Iron, Copper, and Molybdenum complexes with a large shifting in case of Zirconium and Zinc complexes. The different modes of

vibration of C-C, C=C and C-H affected the complex, probably due to the aromaticity of the formed chelate is different from the ligand Malešev and Kuntić (2007). In case of copper complex, the $\nu(\text{C}=\text{O})$ carbonyl stretching is shifted to about 40 cm^{-1} of small peak which ensure the binding of copper metal. In case of iron complexes, the $\nu(\text{C}=\text{O})$ band shifted to sharp peak of lower frequency at 1000 cm^{-1} , $\nu(\text{OCH}_3)$ stretching band shifts to 1000 cm^{-1} and slight shifting of $\nu(\text{O-H})$ band confirms the binding of Fe metal with these groups. But in case of zinc, zirconium and molybdenum complexes there is complete disappearance of $\nu(\text{C}=\text{O})$ band, resulted that metals copper, zinc, and iron binds with oxygen of carbonyl groups. Such data favours that the carbonyl group $\nu(\text{C}=\text{O})$ is strongly affected on coordination with transition (Yaul *et al.*, 2009). In nickel complex the band of phenolic $\nu(\text{O-H})$ and $\nu(\text{OCH}_3)$ stretching found to be shifted which confirms the binding of Ni metal with these groups. These bands are affected on coordination with metal ions, to point that the oxygen atom is a centre for coordination with the metal ions.

Low frequency IR spectra of the metal complexes provide very useful information regarding the type of metal-ligand bond arising from the coordination of ligands to the metal centre. On the other hand, the infrared spectra exhibit weak bands at the range (1050 to 1340 cm^{-1}), which could be assigned to the stretching frequencies of the $\nu(\text{M-O})$. As shown in Fig the characteristic absorption bands of Zn-Cu observed at cm^{-1} which is quite similar to earlier findings. Likewise for zinc complexes the metal to oxygen peak appeared at cm^{-1} supported by earlier studies (Panhwar and Memon, 2014). FT-IR peak at cm^{-1} confirming bonding between copper metal and oxygen. FT-IR of the nickel complex showed bands in cm^{-1} regions that may be attributed to (Ni-O) stretching, new bands in the Zinc complex at 540 cm^{-1} may indicate the formation of metal oxygen (M-O) bond. Infrared absorption frequencies of citrus methanolic extract and bioconjugate of citrus extract. On comparing the spectra of citrus extract with their respective complexes, important distinctions are revealed. The FT-IR spectrum of citrus methanolic extract shows characteristic absorption peaks at 3670 cm^{-1} corresponding to phenolic $\nu(\text{O-H})$, $\nu(\text{C-H})\text{sp}^3$ 3000 cm^{-1} stretching, $\nu(\text{C-H})\text{sp}^2$ 3100 cm^{-1} stretching of flavonoids/phenolics, $\nu(\text{C}=\text{O})$ 1720 cm^{-1} carbonyl stretching of aldehydes or ketenes, $\nu(\text{C}=\text{C})$ 1600 cm^{-1} aromatic ring stretching, $\nu(\text{OH})$ 3600 cm^{-1} alcoholic stretching and $\nu(\text{R-O-R})$ 1000 - 1300 cm^{-1} ether respectively.

A broad band appears in the spectra of the citrus methanolic extract at 87 cm^{-1} which shifted between cm^{-1} in all metal complexes. In copper, zinc, cobalt and iron complexes this band shifted to lower wave number with zinc complex appearing at 3800 cm^{-1} while in case of iron and nickel complexes it shifted to higher frequency. A strong band at about 3566.4 cm^{-1} detected in the spectra of the citrus methanolic extract assigned to $\nu(\text{C}=\text{O})$ stretching

shifted in the spectra of metal complexes indicates that the coordination occurs through the C=O oxygen atom. Complexes of copper, zinc, nickel, and copper causes red shift in $\nu(\text{C}=\text{O})$.

Metal-ligand vibrations are mostly observed below 800 cm^{-1} (Roy, S *et al.*, 2015). FT-IR peaks observed at lower wave number between 450 to 650 cm^{-1} in all complexes, which is not measured in the free ligand spectrum.

4. Conclusions

Plants are the rich source of drugs used in primary health-care. The main advantages of using plants as drugs is safe, low cost and more reliable than the synthetic products; Hence plants can be used as effective pharmacological agents, there is a growing demand for food that is free of synthetic chemicals as preservatives, it is necessary to examine and identify alternatives and safe approaches for controlling food born pathogen. The use of natural compounds from plants could open up the possibility of using them as novel antimicrobials in food system. The results of the present study support the recycling of fruit waste. Thereby, yielding new products and meeting the requirements of essential products required in human, animal and plant nutrition as well as in the pharmaceutical industry.

Acknowledgement

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