Medicinal properties of Cobalt and Copper nanoparticles synthesized using *Limoniaacidissima* leaf extract

.

ABSTRACT

Aim:
Study design: The present investigation was undertaken to synthesize Cobalt and copper nanoparticles (NPs) using *Limoniaacidissima*L. leaf extract and to test their few medicinal properties like antimicrobial antioxidant and anti-diabetic activities.
Place and Duration of Study:*Limoniaacidissima* plant leaves were collected from the vicinity of the College and the study was completed in 11 months.
Methodology: The hot aqueous leaf extract and soxhlet extract using ethyl acetate as solvent were prepared. Phytochemical analysis of the leaf extract showed the presence of Tannins, Flavonoids, Alkaloids and absence of Phenols. The leaf extract was then used to make Cobalt and copper nanoparticles (NPs) using Cobalt acetate and Copper sulphate respectively.
Results: The formation of NPs was checked by color change and confirmed with UV–visible spectrophotometry. The typical peaks of Co-NPs were detected in the range of maximum wavelength between 380-520 nm and that for Cu NPs was detected between 260-580 nm. The antimicrobial activity of the synthesized NPs of both metal oxides was then tested against Gram positive and Gram negative bacteria and also against two Candida sps. Cobalt NPs exhibited strong antimicrobial activity against all tested organisms but Copper NPs showed no antimicrobial activity. The both NPs were also tested for antioxidant and anti-diabetic properties. Comparatively the Copper NPs possessed higher antioxidant and anti-diabetic activity than the cobalt nanoparticles.
Conclusion: Thus, this underutilized plant from India can be further exploited for more medicinal properties and bring it into pharmaceutical usage.

Keywords: [Limoniaacidissima, *Nanoparticles,antimicrobial, antioxidant, anti-diabetic*]

1. INTRODUCTION

Native to india, limoniaacidissima is planted for its fruits across the country's plains, particularly in drier regions. The lovely herb limoniaacidissima has both medicinal and cosmetic uses. Various tree parts, such as the bark, leaves, and fruits, are used in traditional medicine to treat a variety of diseases. Both the ethnomedicinal properties of this plant and the bioactive compounds present in its different sections have been confirmed by recent scientific studies [1].

Chemically produced nanoparticles, sometimes referred to as engineered nanomaterials (enm), carry a toxicity risk when used in biological applications. Using plant extracts is a fairly honest and hygienic way to produce nanoparticles on a large scale. Because of their unique characteristics, nanoparticles can be applied to a wide range of medical applications,

including optical imaging, biological system labeling, antibacterial [2], molecular sensing, and more [3].

Plant biodiversity has been widely taken into consideration for the synthesis of metal/metal oxide nanoparticles because a variety of plant extracts, particularly those from leaves, contain potent phytochemicals like terpenoids, amides, carboxylic acids, flavones, ketones, and ascorbic acids. Metal salts can be reduced by these ingredients to metal nanoparticles [4].

Cobalt nanoparticles have attracted a lot of attention in the last ten years because of their special qualities, which have the potential to be applied in a variety of ways. Physical, chemical, and biological systems are among the strategies for creating nanoparticles that have been reported. Since cobalt is a non-precious alternative to precious metals, its unique catalytic properties have drawn attention from researchers in recent years [5]. Cobalt is a multifunctional semiconductor that is a p-type anti-ferromagnetic semiconductor with a wide range of size-dependent structural, magnetic, electronic, and catalytic properties. Practical uses for this material include heterogeneous catalysis, energy storage, electro-chromic sensors, and anode materials in li-ion rechargeable batteries [6].

For ages, antibacterial and antiviral properties have been attributed to copper and its compounds [7]. Gram-positive and gram-negative bacteria, such as *E. coli*, are susceptible to the antibacterial effects of copper nanoparticles [8].

The antimicrobial properties of silver nanoparticles are well-established [9] and several mechanisms for their bactericidal effects have been proposed. Although only a few studies have reported the antibacterial properties of copper nanoparticles, they show copper nanoparticles have a significant promise as bactericidal agent [10]. However, other nanoparticles, such as platinum, gold, iron oxide, silica and its oxides, and nickel have not shown bactericidal effects in studies with *Escherichia coli* [11]. Yoon et al. 2007 investigated [12] the antimicrobial properties of copper and silver nanoparticles and found that the copper nanoparticles had greater antibacterial activity than the silver nanoparticles.

In this study, we report biological synthesis of cobalt and copper nanoparticles using woodapple plant leaf extract. We carried out antimicrobial, antioxidant and anti-diabetic tests of the biosynthesized nanoparticles.

2. MATERIAL AND METHODS

2.1 Distilled water extraction

About 25 g of leaf was collected, washed thoroughly in distilled water, cut into small pieces and were soaked in 100mL of double distilled water. It was heated in water bath for about 15 minutes at 80^0 C. The resultant extract was cooled down and filtered using Whatman filter paper no.1 and stored in refrigerator for further use as Extract A.

2.2 Extraction Using Soxhlet Apparatus

The dried leaves were crushed and powdered. 25 grams of powdered leaves were consecutively extracted with 250 ml of ethyl acetate in a Soxhlet apparatus at 77°C. Obtained extract was kept for solvent evaporation into open petri-plates overnight. The dark green sticky extract (10 grams) was resuspended in 10ml DMSO and stored at 4°C for further use as Extract B.

2.3 Phytochemicals Analysis

Qualitative phytochemical analyses for the both extracts were performed as described in earlier [13].

2.4 Nanoparticle Synthesis

2.4.1 Cobalt Acetate Nanoparticle synthesis

The aqueous root extract of Limoniaacidissima and 10 mM Cobalt acetate solution were mixed in the ratio of 1:5 and incubated on hot plate at 60°C for 90 min until change in color [14].

2.4.2 Copper Sulphate Nanoparticle synthesis

For nanoparticle synthesis, about 25mL of Wood apple leaf extract was added to 100mL of 1mM copper sulphate solution in a 250mL conical flask. The solution was incubated for a period of 10 hours. Three replicas were placed each at three different temperatures (27°C, 40°C and 80°C). The solution thus obtained was centrifuged at 12000 rpm for 15 minutes. The resultant pellet was washed with distilled water, dried in a hot air oven at 80°C and resuspended in DMSO [15].

2.5 Antimicrobial Activity

Inoculum of test organisms was prepared. 0.1 ml culture of test organisms was evenly spread on sterile Muller Hilton agar plates. Wells were bored carefully in these agar plates using sterile cork borer of 8mm. 100ul of synthesized nanoparticles and positive control was added in respective well. The plates were incubated at 40C for 15 mins, for pre-diffusion and then the plates were shifted to 370C for 24 hrs. Antimicrobial activity was determined by measuring the zone of inhibition [16]. Sterile Potato Dextrose agar plates were used for testing activity against *Candida sps*.

2.6 Reducing power activity

Reducing power assay was determined as described earlier [17]. 1 ml of synthesized nanoparticles was mixed with 1ml of sodium phosphate buffer (pH 6.6) and 1ml of 1% potassium ferriccyanide followed by incubation at 500C for 20 minutes. After adding 1ml of 10% trichloro acetic acid, the mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant was taken out and mixed with 2ml of distilled water and 0.5ml of 1% ferric chloride. After incubation for 10 minutes, the absorbance was measured at 700nm. Higher absorbance of the reaction mixture indicates reductive potential of the extracts. All the tests were performed in triplicates and ascorbic acid was used as reference standard.

2.7 Anti-Diabetic Activity

Anti-diabetic activity of each extract was assessed by alpha amylase inhibitory method. The α-amylase inhibitory activity of extracts was performed using DNSA method with a little modification [18]. Briefly, 1 ml of each solution of extract or standard acarbose (5000 µg/ml) in DMSO was incubated with 1 ml of α-amylase (concentration 3 mg/ml in 20 mM phosphate buffer containing 6.7 mM NaCl, pH 6.9) for 30 min at 37ºC. After pre incubation, 1 ml of 1% starch solution in 20 mM phosphate buffer, pH 6.9, was added. The reaction mixtures were then incubated for 15 minutes at 37ºC. The reaction was stopped by adding 1 ml of DNSA color reagent (96 mM 3, 5-dinitrosalicylic acid and 5.315 M sodium potassium tartrate in 2 M NaOH). The tubes containing resultant mixture were then incubated in a boiling water bath for 5 min and then cooled to room temperature. The absorbance was taken at 540 nm with a UV-Vis spectrophotometer after diluting each tube with 9 ml of deionized water. For correcting background absorbance, the enzyme was replaced by 1 ml buffer solution with similar test procedure. The α-amylase inhibitory activity was calculated by following equation.

α-amylase inhibitory activity Inhibition (%) $= \frac{[(AC+ - AC-) - (AS - AB)]}{} \times 100$

(AC+ - AC-)

Where,

- AC+ represents absorbance of control with 100% enzyme activity (DMSO and Enzyme),
- AC- symbolize absorbance of blank for pure control having 0% enzyme activity (DMSO and Buffer),
- AS represent absorbance of sample or standard (sample/standard and Enzyme) and
- AB symbolize for background absorbance due to sample and standard (sample/standard and Buffer).

3. RESULTS

Phytochemical Analysis

Preliminary phytochemical analysis of leaf extracts of Limoniaacidissima was performed by using various qualitative tests. These tests revealed the presence of Tannins, Flavonoids, Saponins in both the extracts of *Limoniaacidissima*, but at varying intensity. However, Phenols were not detected in both extracts. The results of phytochemical screening of both extracts are shown in table 2.

Table 1: Phytochemical tests for various Limoniaacidissima extracts

Phytochemicals Test	**Extract A** (Hot extract in Distilled water)	**Extract B** (Soxhlet apparatus extract)
Flavonoids	++	+
Alkaloids	+	-
Sapponin	++	+
Tannins	+	++
Phenols	-	-

Characterization of Nanoparticles

By Colour Change

The temporal change in the colour of the mixture of extract and metal indicates the formation of NPs by our plant materials. This is the primary test for the checking of formation of NPs. The reddish brown color of extract A and B with cobalt became intense after incubation (Fig 1a), indicating the reduction of cobalt and thus formation of Co-NPs.

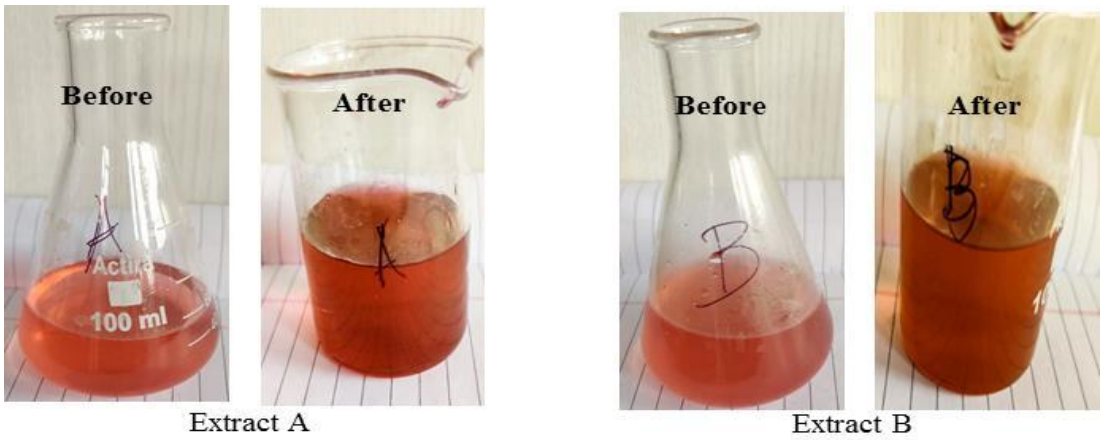


Fig 1a: Cobalt NPs synthesis: Color became intense after 90mins at 60^{0}C for both extracts A and B

The synthesis of Copper NPs was carried out at three different temperatures and 80^{0}C treatment showed intense color change (Fig 1b) indicating the reduction of copper to give Cu-NPs.

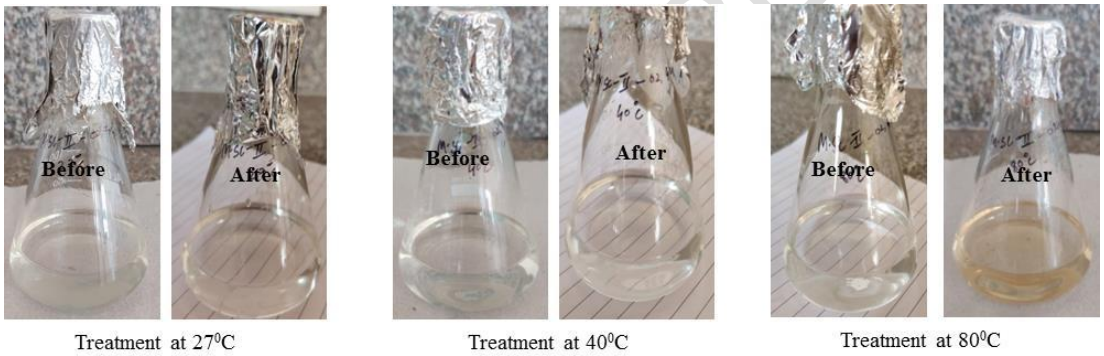


Fig 1b: Copper NPs synthesis with Extract A: Color became intense at 80^{0}C treatment

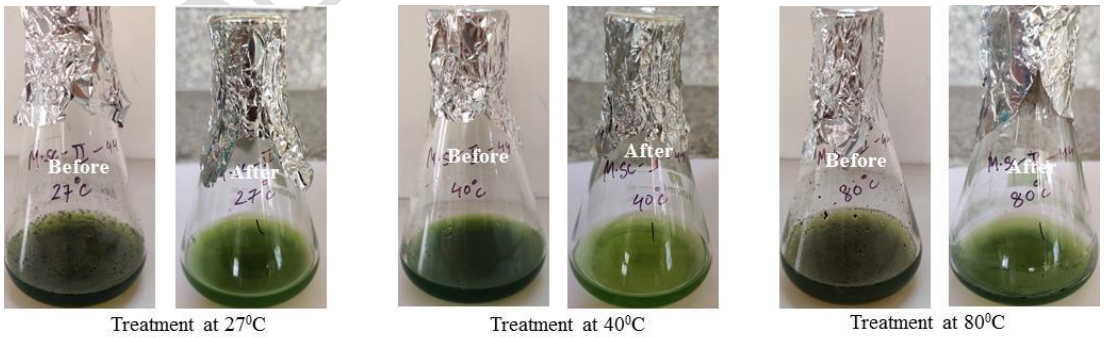


Fig 1c: Copper NPs synthesis with Extract B: Color became intense at 80^{0}C treatment

By UV-visible spectrophotometer

The synthesis of copper and cobalt nanoparticle was first confirmed by UV-Vis spectrophotometer. UV- visible spectral analyses of nanoparticles was done to characterize the NPs formed at a range of 200nm to 700nm. A spectrum of nanoparticles was plotted with

wave length on x-axis and absorbance on y-axis. The λ max for Co-NPs was obtained at 520nm and 382nm for extract A and extract B respectively (Table 2). Similarly for Cu-NPs for extract A it was in the range of 260nm and for extract B in the range of 570nm (Table 2).

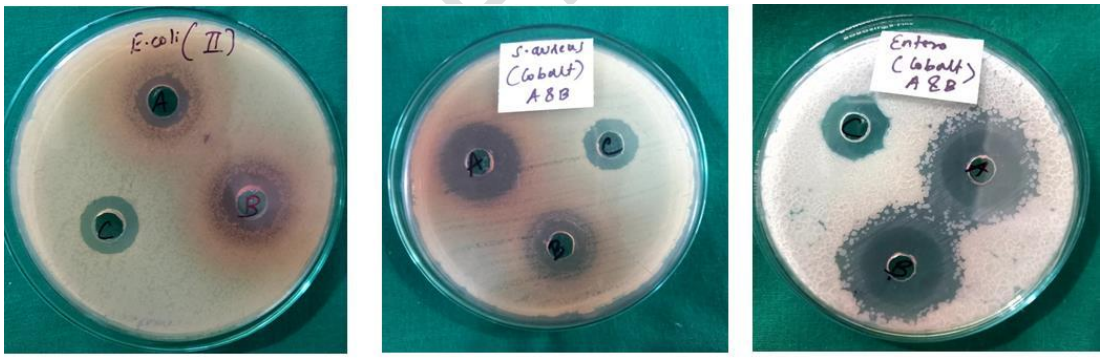
Table 2: UV Spectra for various Nanoparticles

Sr. No.	Extract	Lambda Max
1	Co-NPs Extract A	520nm
2	Co-NPs Extract B	382nm
3	Extract A Cu-NPs [27ºC]	260nm
4	Extract A Cu-NPs [40ºC]	266nm
5	Extract A Cu-NPs [80ºC]	262nm
6	Extract B Cu-NPs [27ºC]	570nm
7	Extract B Cu-NPs [40ºC]	560nm
8	Extract B Cu-NPs [80ºC]	575nm

Note: Extract A- obtained after heating in Distilled water at 80^0C
Extract B- obtained from Soxhlet apparatus extraction method
27^0C, 40^0C, 80^0C treatment temperatures for Co-NP synthesis

Antimicrobial Activity of Cobalt Nanoparticles

Antibacterial activity of synthesized cobalt nanoparticles against Gram negative organisms (*Salmonella, E.coli, , Klebsiella, Shigella*) and against Gram positive organisms (*Staphylococcus aureus, streptococcus mutans, Enterococcus* and *Lactobacillus*) was observed (Fig 2) and zone of inhibition was measured (Table 3). The results indicated that cobalt nanoparticles synthesized from both extracts showed effective antibacterial activity on all tested organisms except *E. coli* and *Klebsiella*. Co-NPs also exhibited significant antifungal activity against *Candida albicans* and *Candida tropicalis*.



E.coli *Staphylococcus aureus* *Enterococcus sp.*

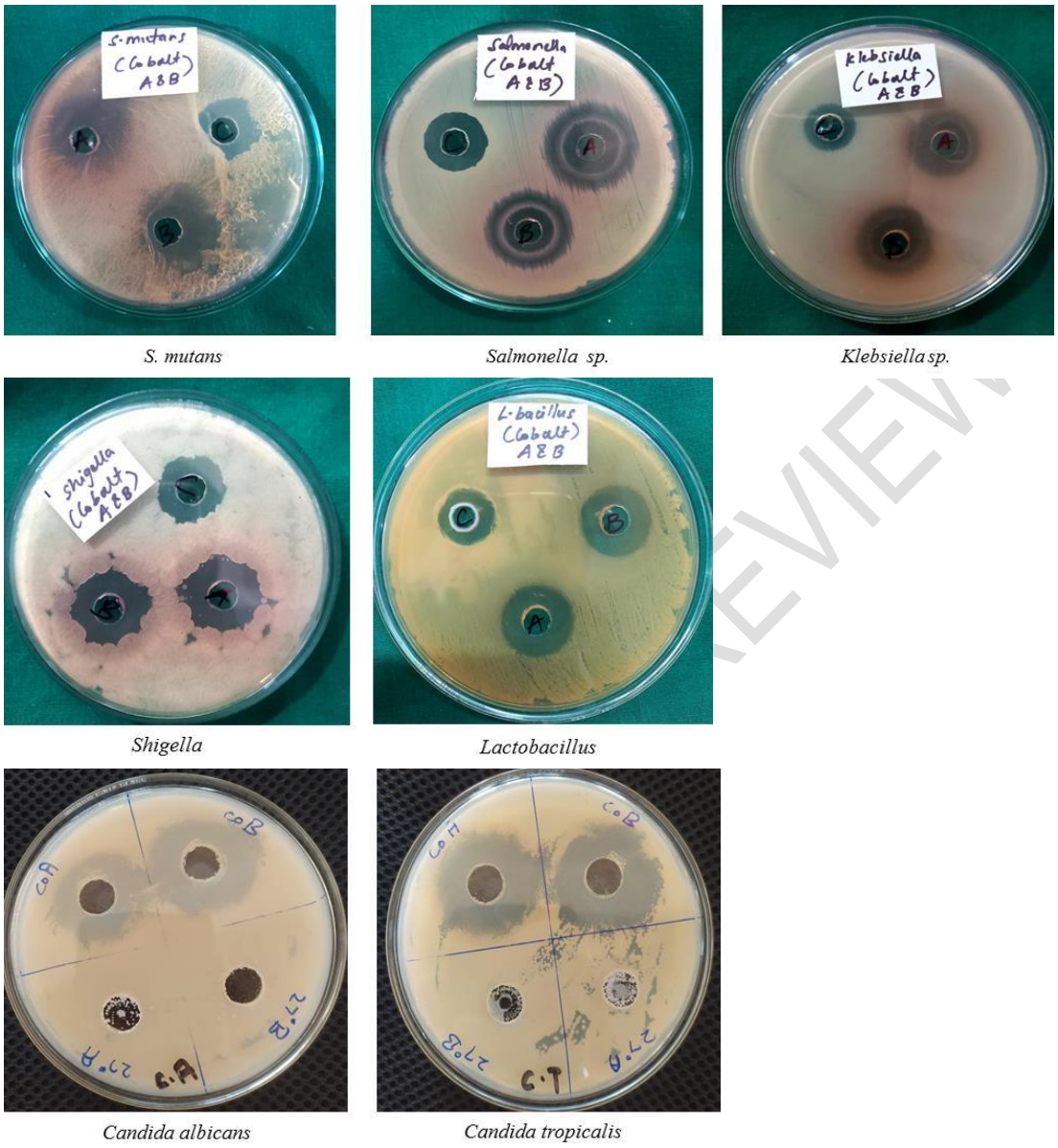


Fig 2: Antimicrobial activity of Co-NPs against test microorganisms

Table 3: Antimicrobial activity of Cobalt nanoparticles synthesized from *Limoniaacidissima*

Sr. No.	Name of test organism	Zone of Inhibition (mm)		
		Co A	Co B	Positive Control
1	*E. coli*	12	13	15
2	*S. aureus*	21	18	15
3	*Entereococcus sp.*	27	27	17
4	*S. mutans*	23	22	17
5	*Salmonella sp.*	26	25	17
6	*Klebsiella sp.*	16	16	14
7	*Shiegella sp.*	23	22	18
8	*Lactobacillus sp.*	21	20	14
9	*Candida albicans*	26	26	-
10	*Candida tropacilis*	27	27	-

Antioxidant activity of Co and Cu nanoparticles

Antioxidant properties and the reducing power of specific plant extracts have a well-established direct relationship. The Cu-NPs from extract B showed higher reducing power than the extract A and Co-NPs from extract A and B. The reducing power of various extracts is mentioned in Table 4. Fe (III) reduction by the extract's electron-donating action is essential to the phenolic antioxidant process.

Table 4: Antioxidant activity of Co and Cu nanoparticles

Sample	Absorbance at 700nm
Co-NPs Extract A	0.033
Co-NPs Extract B	0.249
Extract A Cu-NPs [27ºC]	0.549
Extract A Cu-NPs [40ºC]	0.155
Extract A Cu-NPs [80ºC]	0.714
Extract B Cu-NPs [27ºC]	1.348
Extract B Cu-NPs [40ºC]	1.591
Extract B Cu-NPs [80ºC]	1.671
Ascorbic acid (100µg/ml)	0.530

Anti-diabetic activity of Co and Cu nanoparticles

Enzymes that hydrolyze carbohydrates, such as α-amylase, break down oligosaccharides and digest starch to produce glucose. In order to control hyperglycemia in diabetic patients, one of the main strategies is to limit the activities of α-amylase. The most often prescribed enzyme inhibitor is acarbose. Higher potency to inhibit α-amylase was shown by Cu-NPs with extract A and B than the Co-NPs (Table 5). Percent inhibition of Cu-NPs is nearly comparable with acarbose and thus can be considered as a potential α-amylase inhibitor.

Table 5: Anti-diabetic activity of Co and Cu nanoparticles

Sample	% Inhibition
Co-NPs Extract A	33.25
Co-NPs Extract B	41.86
Extract A Cu-NPs [27ºC]	84.41
Extract A Cu-NPs [40ºC]	80.23
Extract A Cu-NPs [80ºC]	83.95
Extract B Cu-NPs [27ºC]	74.88
Extract B Cu-NPs [40ºC]	86.27
Extract B Cu-NPs [80ºC]	83.48
Acarbose (5mg/ml)	95.85

Discussion

In this present work an attempt was made to synthesize Co and Cu NPs using *Limoniaacidissima* (Woodapple) leaves. Leaf extract of this plant was by two methods; hot aqueous extract and organic solvent soxhlet extraction method [19]. Phytochemical analysis of the leaf extracts showed the presence of Tannins, Flavonoids, Alkaloids and absence of Phenols which was also, determined in a study using *Mimosa pudica* Lin., leaves also showed the presence of alkaloids, flavonoids and tannin [20].

Cobalt and Copper nanoparticals were synthesized using these extracts. In a similar study, *Asparagusracemosus* root extract and Cobalt acetate was used to synthesize Co-NPs. Their

results showed effective antibacterial activity against the pathogenic bacteria[14]. Present study followed the similar protocol to synthesize Co-NPs using *Limoniaacidissima* leaf extract with few modifications as described by a study which used Cobalt nitrate to synthesize NPs [21]. Significant antimicrobial activity of these Co-NPs against all the tested Gram positive and Gram negative bacteria and two Candida sps. was observed. On the same ground, green synthesis of zinc NPs using*Limoniaacidissima* leaves was attempted and they showed that Zn-NPs possessed anti-tuberculosis effect [22].

Ocimum sanctum and Copper sulphate was used to synthesize Cu-NPs that had maximum absorption at 560nm in a study15 by Jayadev and Krishanan, 2021 and the same protocol was followed in the current work to synthesize Cu-NPs that showed their lambda max at 575. Cu-NPs thus synthesized exhibited no antimicrobial activity. On the contrary, Cu-NPs synthesized in a study [23] by Gopalakrishnan et al. 2012 possessed antibacterial activity against *E. coli*.

Antioxidant activity was performed to check the reducing power of Co-NP and Cu-NPsynthesized in present study. In the current work, synthesized NPs also displayed this property, wherein copper NPs showed higher antioxidant activity than the cobalt NPs. On the other hand, studies have determined the antioxidant property of the *Limoniaacidissima* leaf extract [24].

Anti-diabetic activity of the synthesized nanoparticles was carried out. The stem bark [25] and the fruit pulp [26] of *Limoniaacidissima* are known to show anti-diabetic activity but there are no reports of leaf extract of *Limoniaacidissima* showing this medicinal property to our knowledge. Surprisingly the Cu-NPs synthesized from the leaf extract of this plant showed comparable anti-diabetic activity. Acarbose tablet, the standard alpha amylase inhibitor showed 95.58% anti-diabetic property. Copper nanoparticles are having anti-diabetic properties which are in range of the standard drug used but the Cobalt nanoparticles exhibited very less percent of alpha amylase inhibition.

4. CONCLUSION

Globally, the prevalence of diabetes, a metabolic illness, is rising. The regulation of glucose homeostasis is largely dependent on insulin. Protein, fat, and carbohydrate metabolism are all impacted by insulin deficiency [27]. The medical community continues to face difficulties in managing diabetes without adverse effects. It was suggested that inhibiting the activity of this type of alpha-amylase would postpone the breakdown of carbohydrates, which would then result in less glucose being absorbed and a decrease in the rising of blood glucose levels after meals.

In addition to being costly, hazardous, and requiring the use of hazardous chemicals, the chemical processes used to create NP are complex and involve issues like particle aggregation and low stability of the generated nanoparticles [28]. Thus, to conclude the CoNPs and CuNPs synthesized using *Limoniaacidissima* possessed antioxidant and anti-diabetic properties in a similar way as the ZnO NPs synthesized from *Albizzia lebbeck* extract demonstrated in a recent study [29].

CONSENT (WHEREEVER APPLICABLE)

Not Applicable

ETHICAL APPROVAL (WHEREEVER APPLICABLE)

Not Applicable

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