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Medicinal Properties of Cobalt and Copper Nanoparticles Synthesized Using *Limonia acidissima* Leaf Extract

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The present investigation was undertaken to synthesize Cobalt and copper nanoparticles (NPs) using *Limonia acidissima* L. leaf extract and to test their few medicinal properties like antimicrobial antioxidant and anti-diabetic activities.
Methodology: *Limonia acidissima* plant leaves were collected from the vicinity of the College and the study was completed in 11 months. 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

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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: Limonia acidissima; nanoparticles; antimicrobial; antioxidant; anti-diabetic.

1. INTRODUCTION

Native to India, *Limonia acidissima* is planted for its fruits across the country's plains, particularly in drier regions. The lovely herb *Limonia acidissima* 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].

Recently, magnesium, alginate, zinc, copper, titanium, gold, and silver have all been used to synthesize various nanoparticles. Metal nanoparticles may be produced using a variety of biological and physiochemical techniques. Bioreduction is a basic biological process that is employed in the synthesis of metal nanoparticles.

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 (cobalt oxide with the Co_3O_4 formula) 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 a 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 the biological synthesis of cobalt and copper nanoparticles using *Limonia acidissima* (woodapple) plant leaf extract. We carried out antimicrobial, antioxidant, and anti-diabetic tests on the biosynthesized nanoparticles.

2. MATERIALS AND METHODS

2.1 Distilled Water Extraction

About 25 gm of leaf was collected, washed thoroughly in distilled water, cut into small

pieces, and soaked in 100 mL of double distilled water. It was heated in a water bath for about 15 minutes at 80ºC. The resultant extract was cooled down, filtered using Whatman filter paper No.1 and stored in the refrigerator for further use as Extract A.

2.2 Extraction Using Soxhlet Apparatus

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

2.3 Phytochemicals Analysis

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

2.4 Nanoparticle Synthesis

2.4.1 Cobalt Acetate Nanoparticle synthesis

The aqueous root extract of *Limonia acidissima* and a 10 mM Cobalt acetate solution were mixed in a ratio of 1:5 and incubated on a hot plate at 60ºC for 90 min until the color changed [14].

2.4.2 Copper Sulphate Nanoparticle synthesis

For nanoparticle synthesis, about 25 mL of wood apple leaf extract was added to 100 mL of 1 mM copper sulfate solution in a 250 mL conical flask. The solution was incubated for a period of 10 hours. Three replicas were placed at three different temperatures (27ºC, 40ºC and 80ºC). 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 the test organisms was evenly spread on sterile Muller Hilton agar plates. Wells were bored carefully in these agar plates using a sterile cork borer of 8 mm. 0.1 ml of synthesized nanoparticles and a positive control were added to the respective wells. The plates were incubated at 4ºC for 15 mins, for pre-diffusion, and then the plates were shifted to 37ºC 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 1 ml of sodium phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide 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 2 ml of distilled water and 0.5 ml 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.

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

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

3.1 Phytochemical Analysis

Preliminary phytochemical analysis of leaf extracts of *Limonia acidissima* was performed using various qualitative tests. These tests revealed the presence of tannins, flavonoids, saponins in both the extracts of *Limonia acidissima*, but at varying intensities. However, phenols were not detected in both extracts. The results of the phytochemical screening of both extracts are shown in Table 2.

3.2 Characterization of Nanoparticles

3.2.1 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 checking formation of NPs. The reddish brown color of extracts A and B with cobalt became intense after incubation (Fig. 1a), indicating the reduction of cobalt and thus the formation of Co-NPs.

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

3.2.2 By UV-visible spectrophotometer

The synthesis of copper and cobalt nanoparticles was first confirmed by a UV-Vis spectrophotometer. UV- visible spectral analyses of nanoparticles were done to characterize the NPs formed at a range of200nm to 700nm. A spectrum of nanoparticles was plotted with wave length on the x-axis and absorbance on the y-axis. The λ max for Co-NPs was obtained

Table 1. Phytochemical tests for various *Limonia acidissima* extracts

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

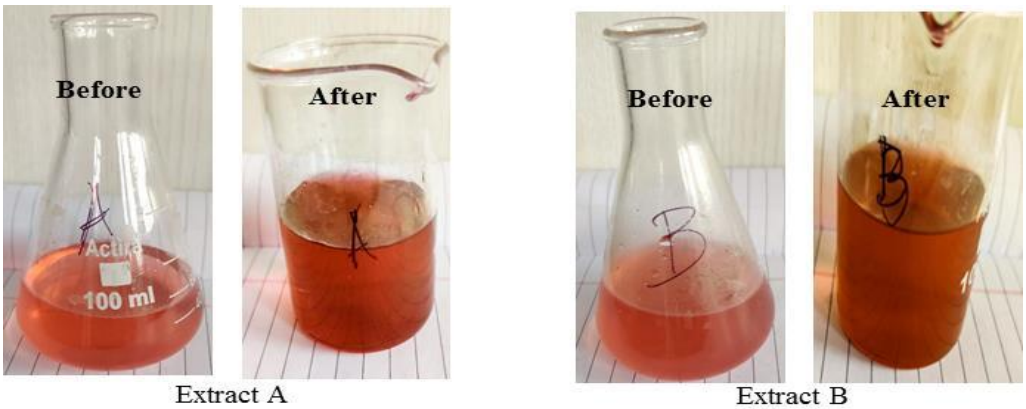

Fig. 1a. Cobalt NPs synthesis: Color became intense after 90mins at 60ºC for both extracts A and B

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

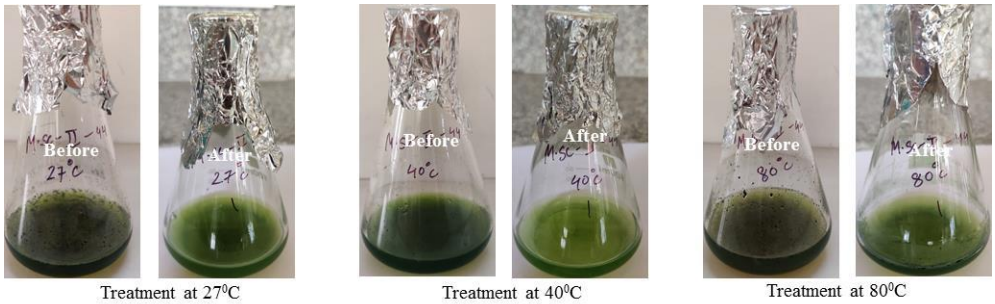

Fig. 1c. Copper NPs synthesis with Extract B: Color became intense at 80^{0}C treatment

at 520nm and 382nm for extracts A and extracts B respectively (Table 2). Similarly, for Cu-NPs in extract A, it was in the range of 260 nm and for extract B, in the range of 570 nm (Table 2).

3.3 Antimicrobial Activity of Cobalt Nanoparticles

Antibacterial activity of synthesized cobalt nanoparticles against Gram negative organisms (*Salmonella, E. coli, Klebsiella,* and *Shigella*) and against Gram positive organisms (*Staphylococcus aureus, Streptococcus mutans, Enterococcus,* and *Lactobacillus*) was observed (Fig. 2), and the 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*.

3.4 Antioxidant Effect 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 extract A, and the Co-NPs from extracts 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 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 80ºC, Extract B- obtained from Soxhlet apparatus extraction method, 27ºC, 40ºC, 80ºC treatment temperatures for Co-NP synthesis

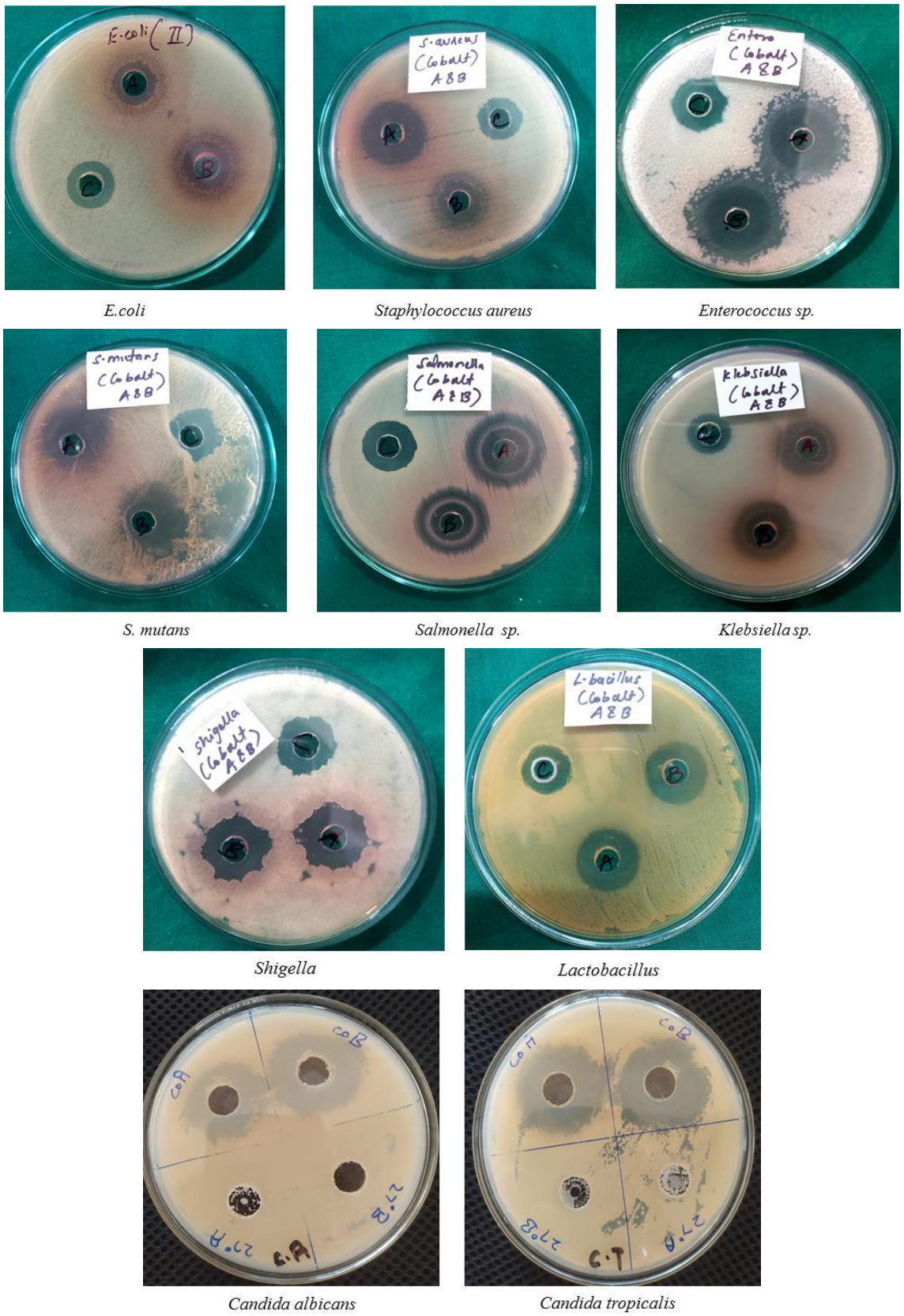

Fig. 2. Antimicrobial activity of Co-NPs against test microorganisms

Table 3. Antimicrobial activity of Cobalt nanoparticles synthesized from *Limonia acidissima*

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	-

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

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

3.5 Anti-Diabetic Effect 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 activity of α-amylase. The most often prescribed enzyme inhibitor is acarbose. Higher potency to inhibit α-amylase was shown by Cu-NPs with extracts A and B than by Co-NPs (Table 5). Percent inhibition of Cu-NPs is nearly comparable with acarbose and thus can be considered a potential α-amylase inhibitor.

4. DISCUSSION

In this present work, an attempt was made to synthesize Co and Cu NPs using *Limonia acidissima* (woodapple) leaves. Leaf extract of this plant was obtained 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 the absence of phenols, which was also, determined in a study using *Mimosa pudica* Lin., leaves also showed the presence of alkaloids, flavonoids, and tannins [20].

Cobalt and copper nanoparticles were synthesized using these extracts. In a similar study, *Asparagus racemosus* root extract and cobalt acetate were used to synthesize Co-NPs. Their results showed effective antibacterial activity against the pathogenic bacteria [14]. The present study followed a similar protocol to synthesize Co-NPs using *Limonia acidissima* leaf extract with a few modifications, as described by a study that 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* spp. was observed. On the same ground, green synthesis of zinc NPs using *Limonia acidissima* leaves was attempted, and they showed that Zn-NPs possessed an anti-tuberculosis effect [22].

Ocimum sanctum and copper sulphate were used to synthesize Cu-NPs that had maximum absorption at 560 nm in a study [15] 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 nm. 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-NP synthesized in the present study. In the current work, synthesized NPs also displayed this property, wherein copper NPs showed higher antioxidant activity than cobalt NPs. On the other hand, studies have determined the antioxidant property of the *Limonia acidissima* leaf extract [24].

The anti-diabetic activity of the synthesized nanoparticles was carried out. The stem bark [25] and the fruit pulp [26] of *Limonia acidissima* are known to show anti-diabetic activity, but there are no reports of leaf extracts of *Limonia acidissima* 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 have anti-diabetic properties that are within the range of the standard drugs used, but the cobalt nanoparticles exhibited a very low percent of alpha-amylase inhibition.

5. 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 metabolisms 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 rise 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 the low stability of the generated nanoparticles [28]. Thus, to conclude, the CoNPs and CuNPs synthesized using *Limonia acidissima* 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].

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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