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Green Synthesis of Iron Nanoparticles from *Moringa Oleifera* Seeds

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Abstract: ***Cold and Soxhlet method of extraction was carried out using Moringa oleifera seeds with aqueous and ethanol as solvents. The qualitative and quantitative analysis was performed which contains phytochemicals like alkaloids, phenols, glycosides, flavonoids, steroids, terpenoids, carbohydrates, proteins and saponins. The nanoparticles were synthesized using ferric chloride at varying ratios and were characterized using UV – spectroscopy, SEM + EDAX, XRD & FTIR. The UV – Spectra peaks showed absorption at 200 – 300nm. The antibacterial activity was performed using E.coli, P.aeruginosa, B.subtilis, S.aureus &S.pyogenes and the antifungal activity using P.chrysogenum, C.albicans &Aspergillus niger. The maximum zone of inhibition against E.coli and C.albicans was measured as 27mm and 32mm. Other applications were performed such as anticoagulation & thrombolytic activity was absent, 86% of antioxidant activity was seen in CEME (Cold Extract M.oleifera ethanol), total antidiabetic activity was higher in CEMW (Cold Extract M.oleifera water), CEME has shown 26.36% of anti inflammatory activity, 71% of water hardness was removed using CEMW extract, CEMW & CEME gave better results for larvicidal activity, 59.76% of heavy metals were removed by CEMW and these were found to be non – toxic to the normal cells using MTT cytotoxicity assay.***

Keywords: Phytochemicals, Green synthesis, nanoparticles, *Moringa oleifera* seeds

1. Introduction

Moringa oleifera Lam. (preferred common names - horse radish tree; drumstick tree; Moringa etc.), belonging to the family Moringaceae. Their leaves, long pods are the edible parts with variety of applications. Each pod consists of fleshy pulp and a round seed with wings or seed kernels. These have high nutritional contents such as proteins, micronutrients, vitamins including carotene and ascorbic acid etc. apart from which these can be used to purify water, produce oils for cooking, biodiesel. The pods and seeds are excellent source of oleic acid (Mono Unsaturated Fatty Acids).

Nanotechnology refers to any substances in the nanometric range (i.e. between 1-100nm). There are mainly two approaches for the synthesis of nanoparticles such as, bottom-up approach (these are chemically synthesized by assembling the smaller building blocks into a larger structure under controlled reaction parameters) and top-down approach (the atoms and molecules are removed from any bulk material to obtain the desired nanoparticles. These are synthesized by lithographic technique and mechanical methods).These approaches requires highly reactive and toxic reducing agents (sodium borohydrate, hydrazine hydrate) causing detrimental impacts to the environment. Thus, the field of green nanotechnology came into existence. These are produced using any plant sources, organisms (such as bacteria, actinomycetes, fungi, yeast etc.) thus making it eco-friendly, more economically reliable with increased surface area, and are easy to scale up.

The iron nanoparticles (FeNPs) has been used for this study as the iron metal is non-toxic, most abundant, easier production, eco-friendly and cheaper. In this paper, several applications of the used green tea leaves – FeNPs (iron nanoparticles) synthesized has been carried out such as anticoagulants / blood thinners (helps in the prevention of blood clots, and used for treating pulmonary embolism, strokes, deep vein thrombosis etc.), thrombolytic agents are clot busters, converting plasminogen into plasmin which breaks down the fibrinogen and fibrin, dissolving the clot. Antioxidant activity prevents the cellular damage due to the over production of reactive oxygen causing cancer, inflammation etc. Heavy metals are those metals which has high density. Some of these in larger amounts can be toxic [such as chromium (Cr), mercury (Hg), thallium (TI) etc.] and cause adverse health effects. Water hardness refers to the amount of calcium and magnesium salts dissolved in the water. The hardness in the water can lead to health effects (malformations of central nervous system; diabetes – due to the higher levels of magnesium, since all the kinases, ATP-related enzymes and channels regulating the insulin action are dependent on magnesium etc.) The typical water softening process uses ion-exchange unit and regeneration process. This requires chemicals such as resin, brine etc., proper maintenance of water softeners and costs are the major limitations compared to the natural adsorbents. In this paper, these problems are being tackled using the green synthesis.

2. Materials and Methodology

2.1. Collection and preparation of the plant materials

The used green tea leaves were collected & preparedfor the extraction (*S.E Abah et al- 2011*)[17].

2.2. Methods of Extraction

Two methods of extraction used in this study are soxhlet and cold extraction using ethanol and aqueous solvents (*S.E Abah et al- 2011*) [17].

2.3. Phytochemical analysis

2.3.1. Qualitative analysis
The extracts prepared were qualitatively analyzed to check the presence of the phytoconstituents using the standard procedure (*S.A Emmanuel et al - 2014*) [16].

2.3.2. Quantitative analysis

2.3.2.1. Determination of Total Alkaloids
The total alkaloid content was estimated according to *Vijay D Tambe et al* (2014) method [21].

2.3.2.2. Determination of Total Phenols
The total phenolic content was determined according to Folin's ciocalteau assay method (*Vijay D Tambe et al – 2014*) [21]. The standard used for calibration was gallic acid.

2.3.2.3. Determination of Total Glycosides content
The cyanogenic glycoside contentperformed using alkaline picrate method *(Nur Faezah Omar - 2012)*[13].

2.3.2.4. Determination of Flavonoids content
The total flavonoid content was performed by aluminium chloride method *(Vijay D Tambe et al – 2014)*[21].

2.3.2.5. Estimation of Steroids
The steroid content was estimated by using potassium hexacyanoferrate (G.Krisgnaveni et al method - 2014)[4].

2.3.2.6. Determination of Total Terpenoids
The total terpenoids content was determined by ammonium molybdate method (Dharmalingam Subha et al - 2015)[2].

2.3.2.7. Estimation of Carbohydrates
The total carbohydrate content was measured using phenol – sulphuric acid method (Neeru Agrawal et al – 2015)[12].

2.3.2.8. Estimation of Proteins
Total protein content was measured by Bradford's method (Marion M. Bradford – 1976) [10].

2.3.2.9. Determination of Saponins content
The total saponins content was determined by vanillin – sulphuric acid method (Raj kumar Tiwari et al - 2016)[15].

2.4 Synthesis of nanoparticles

The prepared extracts were used for the synthesis of iron nanoparticles (Lebogang Katata et al – 2017)[9].

2.5 Characterization

The synthesized nanoparticles were characterized by using UV-Visible spectroscopy, FTIR, SEM & XRD (Lebogang Katata et al – 2017)[9].

2.6. Applications

2.6.1. Antibacterial & Antifungal activity
The antibacterial and antifungal activity was performed based on the standard procedure (Lebogang Katata et al – 2017)[9].

2.6.2. Anticoagulation assay
The anticoagulation assay was performed according to Kartheek Chegu et al (2018)[8].

2.6.3. Thrombolytic activity
Thrombolytic activity was assessed according to Sikandar Khan Sherwani et al (2013)[18].

2.6.4. Antioxidant activity
The antioxidant activity was evaluated using DPPH method (Shalini et al - 2014) [3].
The inhibition percentage was calculated by the formula –
Inhibition (%) = [(Acontrol – Atest) / Acontrol]* 100
Where, Acontrol = ascorbic acid; Atest = absorbance of the samples.

2.6.5. Anti diabetic assay
The anti diabetic activity was measured by DNSA method (M.N. Wickramaratne et al - 2016)[11].

2.6.6. Anti inflammatory test
The anti inflammatory activity was performed according to G. Leelaprakash et al (2011)[5].

2.6.7. Water hardness removal
The removal of water hardness activity was performed by the standard method for measuring the water hardness (Suleyman A. Muyibi et al - 1994)[19]. The percentage of removal was calculated by (C_i = initial hardness (untreated), C_e = final hardness)

$$\% \text{ of removal} = C_i - C_e / C_i * 100$$

2.6.8. Larvicidal test
The larvicidal test was performed according to C. Kamaraj et al (2011)[1].

2.6.9. Removal of heavy metals
The removal of heavy metals was performed by Jeyaseelan et al (2016)[7].

2.6.10. Anticancer activity
The anticancer activity of the samples on VERO and HEP2 cell lines were determined by MTT assay to assess the cytotoxicity(P. Senthilraja et al – 2015)[14].% cell viability was calculated using–
% of viability = Treated – Blank / Control – Blank * 100

3. Results and Discussion

3.1 Collection of plant materials

Figure 1: *M.oleifera* (dried drumstick) seeds

The *M.oleifera* seeds were obtained and were powdered well. These were used for the extraction process.

3.2 Extraction

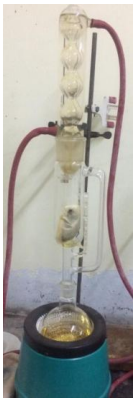


Figure 2: Soxhlet Extraction

Figure 3: Cold Extraction

The soxhlet method of extraction was carried out at 50ºc for 6 hours and cold extraction at 4ºc for 72 hours. These were analyzed for the presence of phytochemicals. Similar procedure was performed using varied weight and concentrations at 40ºc and soxhlet was carried at 80ºc for 8 hours [17].

3.3. Phytochemical analysis

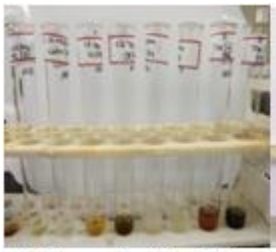


Figure 4: Alkaloids

Figure 5: Phenols

Figure 6: Glycosides

Figure 7: Tannins

Figure 8: Flavonoids

Figure 9: Steroids

Figure 10: Terpenoids

Figure 11: Carbohydrates

Figure 12: Proteins

Figure 13: Saponins

These figures 4 – 13 depict the presence of the phytochemicals in the extract such as alkaloids[21], phenols[21], glycosides[13], flavonoids[21], steroids[4], terpenoids[2], carbohydrates[12], proteins[10] and saponins[15]. This is a preliminary analysis which is essential for quantitative estimation of the phytochemicals.

Table 1: Qualitative Analysis of the extracts

Phytochemical Constituents	Tests	Samples (Solvent extracts)			
		CEMW	CEME	SMW	SME
Alkaloids	Mayer's test	$-^{ve}$	$-^{ve}$	$+^{ve}$	$+^{ve}$
Phenols	$FeCl_3$ test	$+^{ve}$	$++^{ve}$	$+^{ve}$	$++^{ve}$
Glycosides	Conc. H_2SO_4 test	$++^{ve}$	$+^{ve}$	$++^{ve}$	$+^{ve}$
Tannins	Alcoholic $FeCl_3$ test	$-^{ve}$	$-^{ve}$	$-^{ve}$	$-^{ve}$
Flavonoids	10% Lead acetate test	$+^{ve}$	$+^{ve}$	$+^{ve}$	$++^{ve}$
Steroids	Conc. H_2SO_4 test	$+^{ve}$	$++^{ve}$	$+^{ve}$	$+++^{ve}$
Terpenoids	Salkowski test	$+^{ve}$	$+^{ve}$	$+^{ve}$	$+^{ve}$
Carbohydrates	Iodine test	$+^{ve}$	$++^{ve}$	$+^{ve}$	$++^{ve}$
Proteins	Biuret's test	$+^{ve}$	$++^{ve}$	$+^{ve}$	$++^{ve}$
Saponins	Foam test	$++^{ve}$	$-^{ve}$	$++^{ve}$	$-^{ve}$

CEMW - Cold Extraction *M.oleifera* in Water, **CEME** - Cold Extraction *M.oleifera* in Ethanol, **SMW** – Soxhlet *M.oleifera* in Water, **SME** – Soxhlet *M.oleifera* in Ethanol; +^{ve}= Slightly present, ++ ^{ve}=Moderately present, +++ ^{ve}= Highly present,-^{ve}= Absent.

Table 2 shows that Soxhlet extraction gave better results when compared to the cold extraction and ethanol was an effective solvent to extract the phytochemical constituents due to its highly polar nature than aqueous.

Table 2: Quantitative Analysis of the *M.oleifera* extracts (mg/ml)

Solvent extracts	Alkaloids	Proteins	Phenols	Carbohydrates	Steroids	Flavonoids
U1 (CEMW)	-	0.9	2.4	0.42	1.8	1.8
U2 (CEME)	-	1.344	42.6	0.276	0.96	12
U3 (SMW)	139.86	2.5	5	0.8	2.8	2.5
U4 (SME)	170.94	1.625	35	0.2	2.8	18.75

Solvent extracts	Glycosides	Terpenoids	Saponins
M.oleifera extract	0.0058	0.0888	0.756

Table 2 summarizes the results of the quantitative analysis of various phytochemicals. The total protein content was found to be higher in SME (1.625mg/ml) and lower in CEMW (0.9mg/ml). Total carbohydrate content was 0.8mg/ml for SMW (aqueous extract).

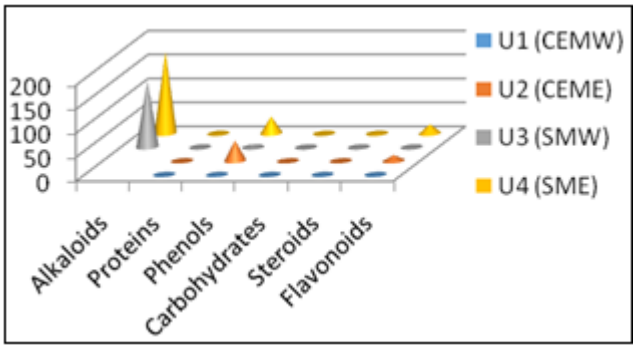


Figure 14: Quantitative analysis of the phytoconstituents

SME has shown higher result (170.94mg/ml) and SMW with lower (139.86mg/ml) for alkaloids. CEME has recorded higher content (42.6mg/ml) and CEMW with lower content (2.4mg/ml) for total phenol. SMW & SME has recorded higher content (2.8mg/ml) for steroids than the aqueous extract. SME has recorded highest (18.75mg/ml) and CEMW has the lowest (1.8mg/ml) for the total flavonoids content. The earlier workers of *M.oleifera* seed extracts have identifiedthe presence of alkaloids, phenols, glycosides, flavonoids, steroids, terpenoids, carbohydrates and saponins only using various solvents such as ethanol, methanol, ethyl acetate and aqueous[16].

3.4 Synthesis of nanoparticles

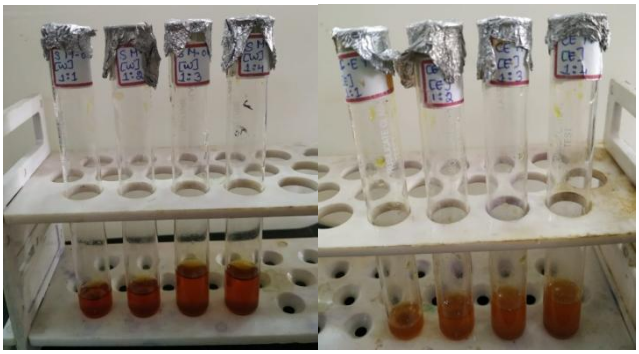
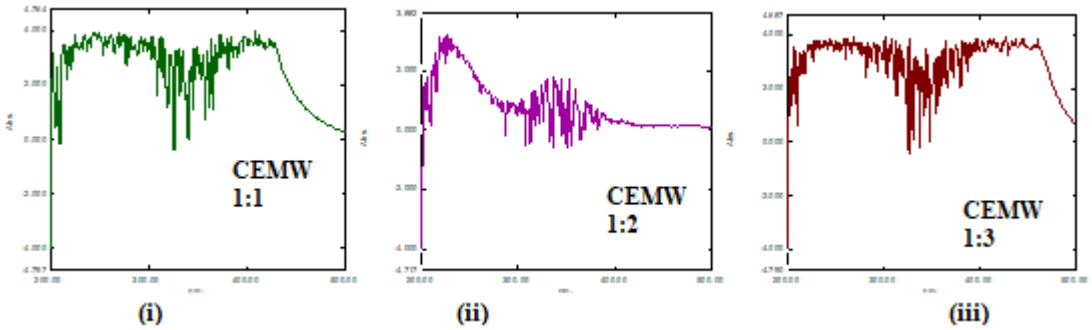


Figure 15: Synthesis of iron nanoparticles (1M of $FeCl_3$ in *M.oleifera* seeds)

The iron nanoparticles were synthesized at varying concentrations (1:1 – 1:4). The colour change from faint yellow to reddish brown and black shows the formation of ironnanoparticles. These were characterized using UV-Visible Spectroscopy, SEM + EDAX, FTIR & XRD. The earlier worker synthesized the iron nanoparticles using defatted seed extractin varying concentrations of 0.1M $FeCl_3$with 1:1, 1:2, 1:3 and 1:4.[9]

3.5 Characterization of nanoparticles

(a) UV-Visible spectroscopy



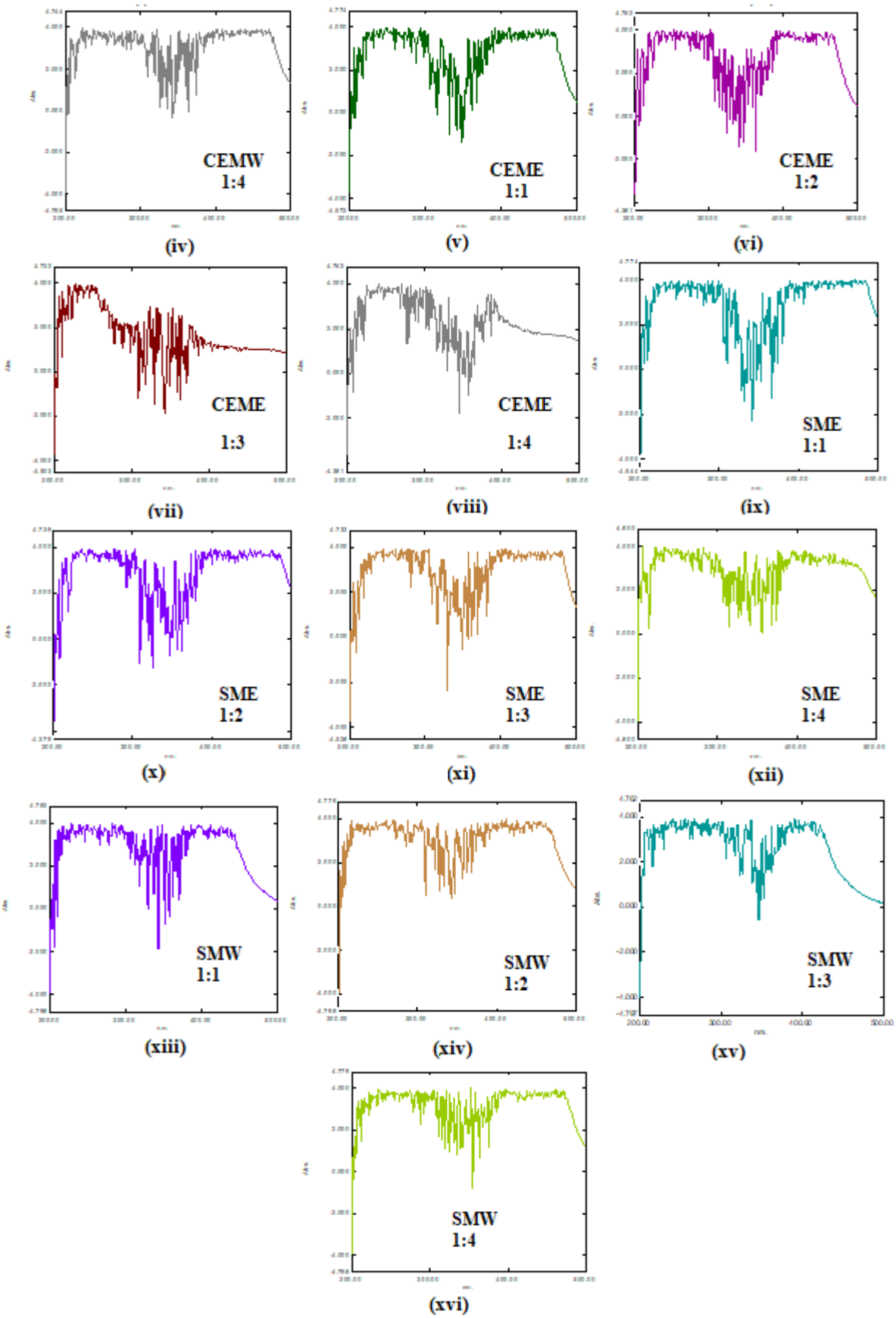


Figure 16: UV – Visible absorption peaks of CEMW FeNPs

This figure 16 shows the UV spectra of CEMW FeNPs. This was measured in the range of 200 – 500nm. The absorption spectrometer works in the range of 200nm (near ultraviolet) to 800nm (very near infrared).

Table 3: UV – Visible spectra measurements

Sample (Extract-FeNPs)	Concentrations	Wavelength (nm)	Absorbance
U1 (CEMW)	1:1	408nm	3.968
		249nm	3.932
	1:2	337nm	1.754
	1:3	427nm	3.895
	1:4	432nm	3.943
U2 (CEME)	1:1	410nm	3.995
	1:2	465nm	3.837
		230nm	4.000
	1:3	402nm	1.340
	1:4	-	-
U3 (SMW)	1:1	422nm	3.662
	1:2	-	-
	1:3	455nm	3.894
	1:4	352nm	3.981
U4 (SME)	1:1	472nm	3.998
	1:2	449nm	3.910
		435nm	3.847
	1:3	406nm	3.960
	1:4	399nm	3.792

The peak formed shows the interaction of the samples (extracts) with the iron nanoparticles. Table 4 describes that CEMW (249nm), CEME (230nm)has lesser wavelengths. Hence both of these were chosen for the further applications. The earlier work was performed with *M.oleifera* seeds and leaves FeNPs. The presence of the nanoparticles was confirmed by the formation of new peak at 240nm. [9]

(b)Scanning– electron microscope

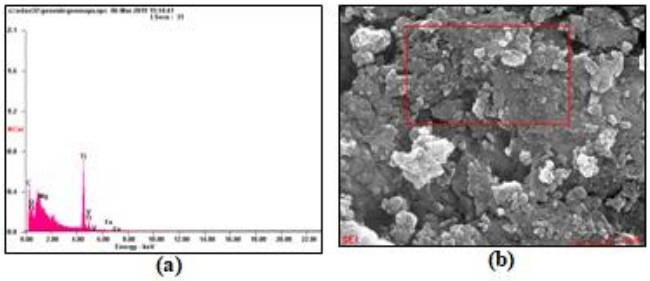


Figure 17: (a) EDX peak (b) SEM image of *M.oleifera* FeNPs

Figure 17 (a) represents the composition of the elements present. EDX results shows the presence of contaminants such as carbon of 56.79% (might be obtained by the use of carbon tape for spreading the powdered samples) and oxygen of 25.10% along with other constituents such as magnesium (01.72%), Tin (16.02%), iron (00.23%) and vanadium. The SEM image shows the formation of aggregated nanoparticles with irregular shapes and size. The earlier work was performed with *C. sinensis* leaves FeNPs; it had an average diameter of about 116nm.[6]

(c) FTIR

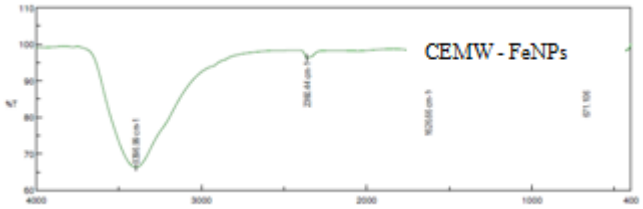


Figure 18: FTIR spectra of CEMW – FeNPs

Figure 18 shows the FTIR spectra of CEMW – FeNPs displaying the stretching at 3396.99 cm^{-1} for N-H stretching, at 2360.44 cm^{-1} for O=C=O stretching, at 1626.66 $cm^{-1}$for the C=Cand the formation of band at 671.106 cm^{-1}confirms the presence of the FeNPs.The earlier worker performed with *M.oleifera* seeds and leaves FeNPs which showed the bandsstretching at 3519 $cm^{-1}$for O-H, at 1615 $cm^{-1}$for the C=C and 1049 $cm^{-1}$for C-O-C absorption peak and formation of FeNPs was confirmed by a band at 567 cm^{-1}.[9]

(d) XRD

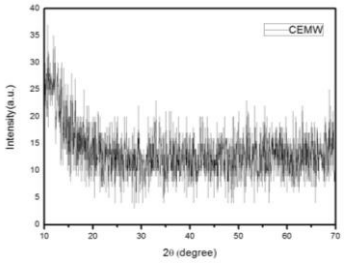


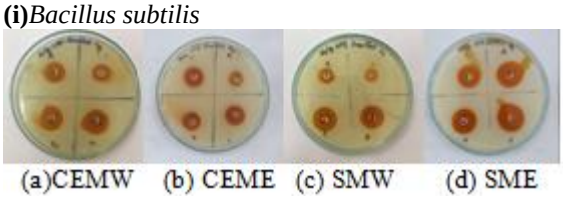
Figure 19: XRD pattern for CEMW – FeNPs

In Figure 19, noise was observed which can be due to fluorescence of an element in the sample thus enhancing the background etc. The earlier work in *M.oleifera* seeds and leaves FeNPs showed peak around 2θ of 45° on MOS – FeNPs [9].

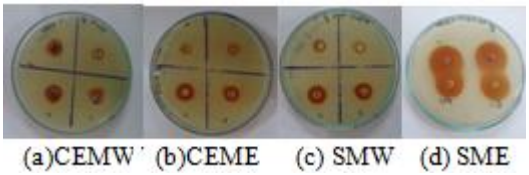
3.6. Applications

(a) Antibacterial activity

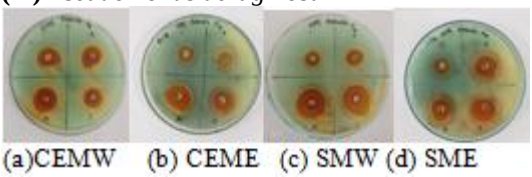
(i)*Bacillus subtilis*



(ii)*Escherichia coli*



(iii) *Pseudomonas aeruginosa*



(iv) *Staphylococcus aureus*

(a) CEMW (b) CEME (c) SMW (d) SME

(v)*Streptococcus pyogenes*

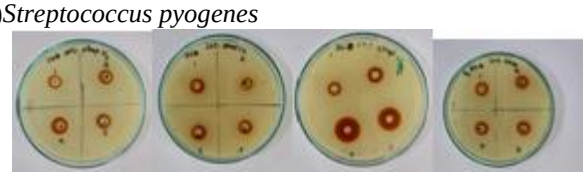


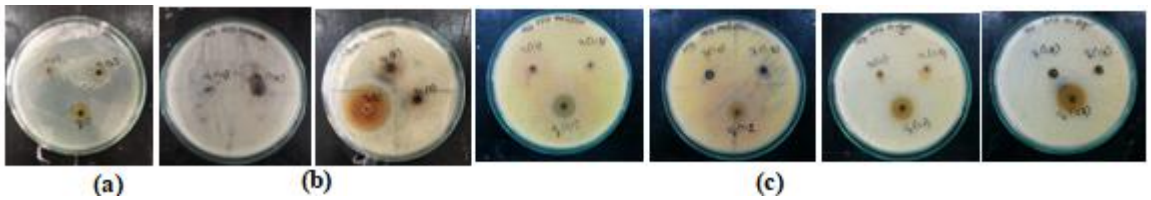
Figure 20: Antibacterial activity

Table 4: Zone of inhibition (in mm) of *M.oleifera* FeNPs

Sl.No.	Organisms	Concentration (in ratios) Zone of inhibition (in mm)															
		CEMW				CEME				SMW				SME			
		1:1	1:2	1:3	1:4	1:1	1:2	1:3	1:4	1:1	1:2	1:3	1:4	1:1	1:2	1:3	1:4
1	*B. subtilis*	14	16	18	20	17	12	17	18	10	13	19	20	19	20	22	20
2	*Escherichia coli*	9	10	13	15	9	8	12	14	9	10	13	15	26	27	24	21
3	*Pseudomonas aeruginosa*	17	16	16	18	11	12	16	20	11	14	20	21	12	17	17	15
4	*Staphylococcus aureus*	17	20	20	21	20	15	18	20	13	17	19	17	16	16	16	16
5	*Streptococcus pyogenes*	10	11	11	12	11	10	11	12	10	11	17	18	11	12	12	12

This shows that the SME FeNPs (22mm) showed higher zones for *B. subtilis*. Ethanolic extract of *M.oleifera* (SME - 27mm) at 1:2 showed susceptibility for *E.coli*.SMWand CEME (20mm) showed the maximum activity for *P. aeruginosa*. CEMW (21mm) at 1:4 showed maximum zone for *S. aureus*. *S. pyogenes* was more susceptible to SMW (18mm) at 1:4. Mostly soxhlet extracts gave better zone of inhibition.The earlier worker performed with *M.oleifera* FeNPs and different antibiotic discs against *E.coli*[9].

(b) Anti fungal activity



(a) (b) (c)

Figure 21: Anti fungal activity (a)*Candida albicans*, (b) *Penicillium chrysogenum*, (c) *Aspergillus niger*

Table 5: Zone of inhibition for Anti- fungal activity

Organisms	*Zone of Inhibition (mm)*	
	U3(1:1) CEMW	U7 (1:2) CEME
Penicillium chrysogenum	17mm	15mm
C.albicans	12mm	32mm
Aspergillus niger	16mm	19mm

This clearly shows that *P.chrysogenum*, *C.albicans* and *A.niger* were susceptible to these samples – FeNPs. CEMW (1:1) showed higher zones (17mm) for *P.chrysogenum*. CEME showed higher zone (19mm) for *A.niger*.The result revealed that *M.oleifera*FeNPswere effective against*C.albicans* for CEME (32mm).The earlier worker performed only with *C.albicans* for *Anchomanes difformis* leaf extracts [17].

(c) Anticoagulation assay

Thisstudy showed that (U3) CEMW & (U7) CEME FeNPs gavenegative results. The earlier worker performed using *Allium sativum, Zingiber officinale.* [8]

(d)Thrombolytic activity

Thisstudy that *M.oleifera*extracts FeNPs (i.e. CEMW & CEME) did not exhibit the activity. Earlier worker performed using streptokinase as reference standard for aqueous and methanolic crude extracts of *Camellia sinensis* which exhibited the thrombolytic activity [18].

(e)Antioxidant activity

Table 6 depicts that the CEME (at 1:2) showed higher anti oxidant activity of 86%. The earlier worker performed using green and black tea in which higher level of activity (0.08mg/ml) using methanol and ethyl acetate solvents was found [3].

Table 6: % of inhibition for DPPH-scavenging activity

Samples	% of Inhibition
U3 1:1 (CEMW)	73.5%
U7 1:2 (CEME)	86%

(f)Anti diabetic assay

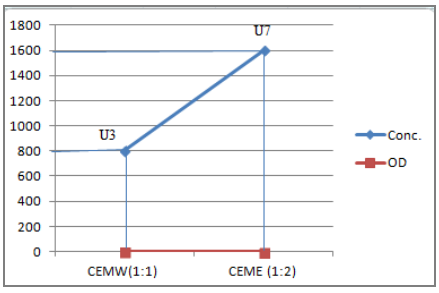


Figure 22: Anti diabetic activity of the FeNPs extract

Table 7: Anti diabetic activity of the FeNPs extract

S.No.	Extracts – FeNPs (sample)	Total activity of the sample (in IU)
1	U3 1:1 (CEMW)	900,000
2	U7 1:2 (CEME)	41,666.66

Figure 26 and table 7 shows that the highest activity was observed in CEME with 41,666.66 IU. The earlier worker performed using *Adenanthera pavonina* leaf extracts. This study showed the in vitro assay of alpha amylase inhibitory activity[11].

(g) Anti inflammatory test

Table 8: Anti inflammatory activity

Samples	% of Inhibition
U3 1:1 (CEMW)	23.16%
U7 1:2 (CEME)	26.36%

Table 8shows the highest % of inhibition at CEME (26.36%) at 1:2.The earlier worker performed using methanolic extract of *Enicostemma Axillare*. The results showed that the sample exhibited this activity at varying concentrations [5].

(h)Water hardness removal

Table 9: % of water hardness removal

Samples	*% of Removal*
U3 1:1 (CEMW)	71%
U7 1:2 (CEME)	NIL

Table 9 shows that water hardness was more efficiently removed by CEMW – FeNPs (71%). The earlier work performed by using *M.oleifera* seeds for treating surface water, synthetic water and ground water which gave better results at higher dosage[19].

(i)Larvicidal activity

Table 10: Larvicidal activity of the *M.oleifera*extracts – FeNPs

Sl. No.	Extracts – FeNPs	Time duration (in minutes)
1	CEMW (1:1)	30 minutes
2	CEME (1:2)	After 30 minutes

Table 10 shows that CEMW – FeNPs was more effective. Earlier worker performed against *Anopheles subpictus* &*Culex tritaeniorhynchus* using methanol, ethyl acetate and acetone extracts of *A.squamosa* L., *C.indicum* L., and *Tridax procumbens*[1].

(j) Removal of heavy metals

Table 11: % of heavy metals removal

Samples	% of Removal
U3 1:1 (CEMW)	59.76%
U7 1:2 (CEME)	59.17%

Table 11 shows that CEMW (59.76%) removed the heavy metals efficiently. The similar work was performed using green tea leaves for the removal of chromium [7].

(k) Anticancer activity

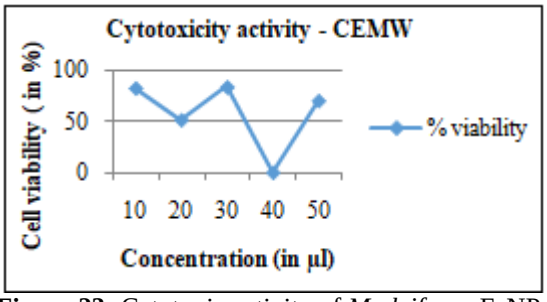


Figure 23: Cytotoxic activity of *M.oleifera*– FeNPs

Table 12: % of cell viability

Samples	% of Cell Viability				
	10µl	20 µl	30 µl	40 µl	50 µl
CEMW (1:1)	83.33%	51.97%	84.46%	-	70.90%

Figure 23&Table 12 shows that the FeNPs extracts were non- toxic to the VERO III cells, and didn't have any anti-cancer property. The % of viability was higher in CEMW having 84.46% of viable cells at 30 µl. Similar work was performed using marine yeast by MTT assay in Vero, HepG2 and MCF -7 cell lines[14].

4. Conclusion

The extracts were prepared from *Moringa oleifera* seeds by cold and soxhlet method of extraction. The extraction process was carried out using ethanol and aqueous solvents. The phytochemical analysis was done qualitatively and quantitatively which showed the presence of different phytochemicals such as alkaloids, flavonoids, glycosides, steroids, terpenoids, proteins, carbohydrates, saponins and phenols. The iron nanoparticles were synthesized in varied concentrations andthis was characterized using SEM + EDAX (Energy Dispersive X-Ray Analysis), UV- Visible spectroscopy, FTIR (Fourier Transform Infrared Spectroscopy) & XRD (X-Ray Diffraction). The presence of nanoparticles was confirmed using the morphological shape, size, its chemical composition etc. These synthesized nanoparticles were used for several applications such as removal of water hardness and heavy metals, antibacterial, antifungal, anti coagulant, anti oxidant, thrombolytic, anti diabetic, larvicidal, anti inflammatory and anti cancer activities showing positive results.

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