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Research Article

Reverse Phase HPLC Method Development and Authentication for Qualitative and Quantitative Assessment of Quercitin, Rutin and Chlorogenic Acid in Morus Indica Leaf Extract.

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Abstract
Morus indica is a woody perennial tree with a high concentration of active medicinal components. It is widely cultivated for its flavor , nutrition and medical importance. Economic importance of Mulberry is a well known. Currently there's a major threat to the living world and public well-being due to rapidly rising of harmful pollutants in air, soil and ground water. Mulberry plant gives promising result in mitigating the harmful impacts of heavy metals in polluted soils, as it has deep root system and suitable environment adaptation and mulberry leaf have strong absorption capacity in reducing air pollution. Quercitin, isoquercitin, quercitrin, isoquercitrin, rutin, chlorogenic-acid and additional flavanoids can be observed in their leaves. The crucial elements of combined flavanols in the leaves of *Morus indica* are rutin, quercitin and chlorogenic acid. The goal of Reverse Phase HPLC proposal was to design and corroborate a procedure for recognizing these elements. HPLC (Shimadzu technologies LC series) with a UV--Vis detector and C18 column (250mm, 4.6mm, 5µm particle size) was utilized. For quercitin, rutin, and chlorogenic acid the devised method produced high authenticity, high validity, high observable, high robustiousness, high strength and strong linearity. The present research aimed to successfully standardized the tissue culture protocols and employ them in the improvement of *Morus indica* plant in vitro.

Keywords: Mulberry, Plant Tissue Culture, Quercitin, Rutin, Chlorogenic acid, RP-HPLC.

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Introduction

Morus indica commonly recognize as Indian Mulberry, which is a species of mulberry widely cultivated under diverse weather conditions from moderate to sub tropical region, particularly in India, for its leaves, which is supposed to be only food for silkworm (Bombyx mori) in the field of Sericulture. At present mulberry is cultivated on 282,244 hectares in different states of India. Today around 80% of mulberry seedlings are reproduce vegetatively in India. (Dutta, *et.al.*, 2002). Only 16.1% of silk production occurs in

India (Taha, *et al.,* 2020). Various researchers has been active on vegetative multiplication for centuries and give various technique to improvise the cultivation of mulberry. (Giacomin, 2017). In order to produce high yielding and suitable to various environmental condition, improved varieties of mulberry should be propagated in vitro. Biotechnology interventions through Plant tissue culture for *Morus indica* (Mulberry) has made a significant strides over the past few decades. Furthermore, various techniques provided by the plant tissue culture for the expansion of genetically high-caliber mulberry varieties with desirable trait such as leaf size increase, disease resistance and stress tolerance, practical applications are like genetic improvement with desirable trait; rapid propagation ensures uniform quality and rapid production of disease free mulberry plantlets; conservation of germplasm to preserve rare and endangered Morus indica genotypes; enhanced production of secondary metabolite found in mulberry such as flavonoids and anthocyanins; somaclonal variation to generate novel mulberry variants with unique characteristics; genetic transformation to generate transgenic varieties with improved traits; protoplast fusion to generate hybrid varieties of mulberry plants; haploid production to produce homozygous lines; somatic embryogenesis for improving efficacy in commercial cultivation and cryopreservation for prolonged storage of mulberry germplasm (Titanji, *et al.,* 2007; Barnes *et al.,* 2008; Rai *et al.,* 2009; Bhatti and Jha, 2010). Today, Mulberry is considered as a all-purpose plant due to its part in environmental sustainability, for its medicinal value and its use in various industrial applications across multi sectors such as ayurveda and nutritive values for advancement of human health; industrial applications for processed products; animal husbandry, bio-fuel production and many more. Indian Mulberry is also considered as a distinctive plant on the planet due to its vast adaptability under diverse climate conditions, soil conditions and 4000m above mean level of sea (Ercisli and Orhan, 2007; Khan *et al.,* 2013; Sarkar et al., 2017).

The main aim of this study is to successfully propagate the *Morus indica* plant *in vitro* and design the method for authentication of three elements i.e., Quercitin, Rutin and chlorogenic-acid in the leaves of *Morus indica* by employing RP-HPLC method are also discussed. In the present study, an efficient protocol for inducing direct organogenesis of *Morus indica* from explants is presented.

2. Materials and Methods.

Plant Collection and Explants disinfection

Thriving *Morus indica* (MI) twigs were collected from the local area of Dehradun, Uttarakhand region. Nodal segments with axillary buds were excised and cleaned with a soft brush and tap water and shaken in 2% tween-twenty solution for 5mins (Himedia,Mumbai, India). After which three rinses in distilled sterile water were done. Explants were subjected to surface sterilization with 70% ethanol (EtOH) for 1 min., followed by 1-3% mercuric chloride ($HgCl_2$) for 3 minutes and 1-3% sodium hypochlorite (NaOCl) for 5 minutes and then they were cleansed three times with sterile double demineralized H2O for one minute each. Next, the cut ends were properly trimmed with a disinfected scalpel and cultivated in the culture media vertically inside the aseptic conditions of laminar airflow cabinet (Teixeira da Silva *et al.,* 2016, da Silva *et al*., 2016).

Medium composition and culture conditions

Murashige and Skoog media were applied for every stage of tissue culture. (Saini *et al.,* 2023).The M.S. medium were complemented with myoinositol (100 mg/l), glycine(2 mg/l), nicotinic acid(0.5mg/l), pyridoxine (0.5mg/l), and thiamine hydrochloride(0.10mg/l), (MS vitamins) with sucrose 3% (w/v) and agar 0.8%(w/v) (Himedia, Mumbai, India). 1N HCL and 1N NaOH were added after the growth regulators were added to lower the pH to about 5.8.The medium were disinfected using autoclave for 15mins. at 121°C . For the process of shoot development, 25ml by 150mm culture tubes were used along with 25ml of M.S. media and cultured pots were secured with cotton gauze plugs. The cultures were placed in a growing environment with the following parameters: 40µmolm-2/s, 25±2°C, relative humidity is set to 75% and brighten with white fluorescent lights (15W, ED, India) with a 16 hour photoperiod (Gaurav *et al.,* 2018)

Multiplication and Elongation of Shoot

Bud break and shoot development were achieved by infected the plants with 6 benzyl amino purine (BAP;0.5to2.5 mg/l), kinetin(kin; 0.5to2.5 mg/l) and adenine-sulphate (ADS;10to40 mg/l) alone or in combination (Himedia, Mumbai, India). After the first 30days, individual shoots that reached 2-3cm in height had been collected and endorsed to develop on M.S. medium complemented with 1.5mg/l Gibberellic acid. (Peddaboina, *et al*, 2006, Wochok ZS, *et* al., 1980). The shoots were left to further incubate for additional 14 days. Regular checking for indicators of contamination as well as changes in physiology and morphology.

Rooting

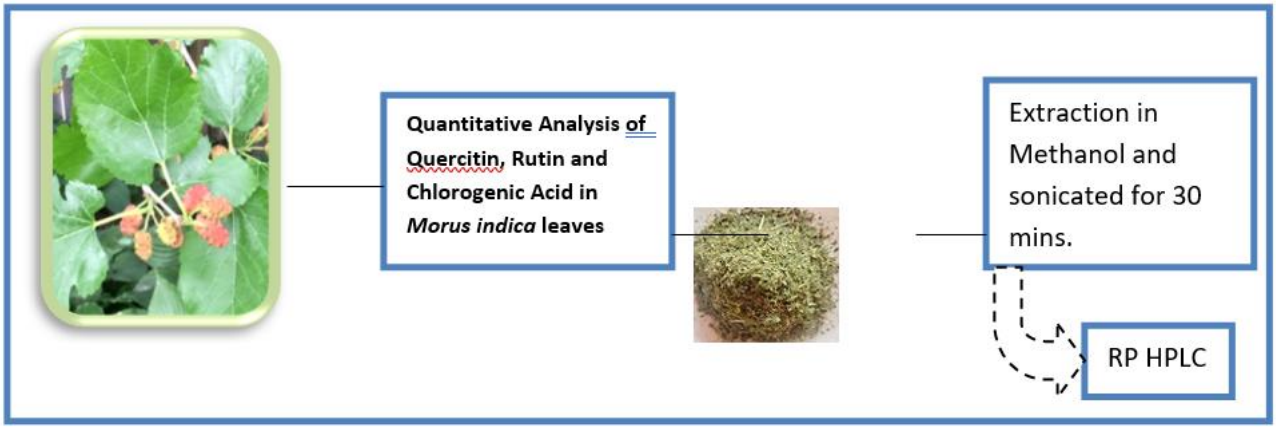
M.S. medium accompany with 0.5% (w/v) activated charcoal and 1Napthalene-Acetic Acid (NAA;0.5mg/l), Indole- Butyric Acid (IBA; 0.5 mg/L), Indole-Acetic Acid (IAA; 0.5 mg/L), single shoots with a height of 4-6cm were developed. A two phase approach was used to analyze the rooting. Micropropagated shoots were cultivated for 1-9 days on M.S. media with different ranges of 2,3-D (0.1-2 mg/l) (De Klerk G-J, 2002). When the shoots began to show indications of root initials, they were moved to 1/2 strength MS hormone free culture medium to promote growth of roots and multiplication. (Dubey V. *et al.,* 2020)

Acclimatization

After the removal of roots from the culture flasks, the plants were cleaned thoroughly with tap water to get rid of any agar. After that, the plantlets were transferred in to circular 500mL garden pots that were filled with a 1:1:2 mixture of autoclaved field soil, sand and vermi-compost and coated with see-through polythene bags. Potted transplanted plant were nurtured in the incubation room at 25±2°C (40µmol, m^{-2} s^{-1}) with photoperiod of 16hours . The relative humidity were lowered to 60% over the course of next 6 days after being set at 75% for 4days using dehumidifier (Loba, New Delhi, India) (Zapata, *et al*., 2003, Bag N, *et al*., 2019) Before the planting in the field, the plants were developed in the culture chamber for 15days. After that, they were moved to a green house for three weeks to allow additional growths. Following a four week period the survivorship of plants were noticed.

Qualitative and Quantitative determination of bioactive marker RP-HPLC method.

The RP- HPLC method has been established for the evaluation of three marker compounds in the *in vitro* raised *Morus indica* extract, using C18 reverse phase column. The marker compounds viz., Quercitin, Rutin and Chlorogenic acid were quantified in *in vitro* raised *Morus indica* leaf extract by HPLC method.



Extraction of Plant

One kilogram of fresh *Morus indica* leaves were dehydrated under shade at room temperature of about 25°C for about 20 days. Plant parts were crushed into a fine powder using a machine. 100g of dried leaf powder were saturated in Methanol and sonicated for 30 minutes to extract the targeted marker compounds. Extracted marker compound were obtained by filtering the solution through 0.45 µm membrane filter. (Zhang, *et al*., 2016).

RP-HPLC

Multiple analytical procedures were employed for the assessment of biological samples, pharmaceutical formulations and sample of drugs in bulk**.** HPLC (High Performance Liquid Chromatography) is a chromatographic technique for the identification, quantification and separation of number of components mixture present in the sample. HPLC utilized in the field in analytical chemistry and biochemistry. RP HPLC is the most extensively used technique for analytical and primary separation of compound of concern. Reverse Phase technique of HPLC has been utilized for biological separation such as to separate components with diverse degree of hydrophobicity like proteins, peptides and nucleic acids with satisfying retrieval and resolution. (Girase, *et al.*, 2024).

HPLC Conditions for Marker Compound

Quercitin: C18 reverse-phase column were used for estimation of Quercitin. The mobile phase consists of a composition of acetonitrile, (0.1%) formic acid in water, in a specific gradient or isocratic ratio (e.g., 60:40), ensuring proper separation of quercetin from other sample components, at flow rate of about 1.0ml/min and observed retention time of 1-9min with sharp points. Detection was performed at the 370nm (optimal for detecting quercitin) (Garg, *et al.,* 2023).

Standard Calibration Curve: A calibration curve for quercetin was prepared by injecting known concentrations of the standard quercetin, ranging from 50 µg/mL to 300 µg/mL. Each concentration was introduced into HPLC complex, and area of peak were recorded.

Rutin: C18 reverse-phase column were used for estimation of Rutin. The mobile phase consists of a composition of methanol with water (acidified with 0.1% orthophosphoric acid) in a specific gradient or isocratic ratio to ensure proper separation of rutin from other sample components, at flow rate of about 1.0ml/min and observed retention time at 1-9min with sharp points. Detection was performed at the 254nm (optimal for detecting quercitin) (Chan, *et al.*, 2016).

Standard Calibration Curve: A calibration curve for Rutin was prepared by injecting known concentrations of the standard Rutin, ranging from 50 µg/mL to 300 µg/mL. Each concentration was introduced into the HPLC system, and the peak area were recorded.

Chlorogenic Acid: C18 reverse-phase column were used for the estimation of Chlorogenic Acid. The mobile consists of a composition of methanol with

water (acidified with (0.1%) formic acid) in a ratio 60:40 (v/v) to ensure proper separation of Chlorogenic Acid. from other sample components, at a flow rate of 1.0ml / min and observed retention time at 1-60 min with sharp points. Detection was performed at the 327nm (optimal for detecting Chlorogenic Acid). (Aiyarakanchanakun, 2021) (Zhai, *et al*., 2018).

Standard Calibration Curve: A calibration curve for Chlorogenic Acid. was prepared by injecting known concentrations of the standard Chlorogenic Acid, ranging from 50 µg/mL to 300 µg/mL. Each concentration was introduced into the HPLC complex, and area of the peak were recorded.

Statistical Analysis

The distribution of *Morus* samples and their marker compounds were analyzed. Tukey's multiple range test was utilized to evaluate the statistical significance mean difference between the different treatments at $p<0.05$. For data visualization, the mean ± Standard Deviation (SD) was employed. Graph Pad Prism 8 (GraphPad Software, USA) applied for statistical analysis.

RESULTS AND DISCUSSION

Culture Induction, Shoot A mplification, Rooting and Acclimatization

Initiation of bud break was observed on the seventh day. The ideal setting for shoot commencement was kin concentrations. At 1mg/L Kin, the maximum shoot length, leaf number, and internode number were observed. In comparision with traditional method, a 2phase rooting method using 2,4-D produced more root proliferation and shorter culture time. After 28 days period of cultivation during the rooting phase, the plantlet indicated robust root and shoot systems. (Table 1). Following that, plantlets were transferred in garden pots with a plastic bag to keep high humidity level. 1:1:2 autovclaved combination of field soil, vermicompost and sand was added in the garden pots.

Table 1: Effect of plant hormones used on different plant improvement parameters such as shoot initiation, multiplication and rooting.

Plant Growth Regulator	Concentration (mg/L)	Shoot Initiation	Shoot Multiplication	Rooting	Other Observations
BAP (6-Benzylaminopurine)	0.5	+++	++	-	Enhanced shoot length
BAP	1.5	++++	+++	-	Increased number of shoots
BAP	2.5	++++	++++	-	Optimal for shoot proliferation
Kinetin	0.5	++	++	-	Moderate shoot initiation
Kinetin	1.5	+++	++	-	Enhanced leaf development
Kinetin	2.5	+++	+++	-	Increased shoot length
Adenine sulphate (ADS)	15	-	-	++	Induced root initiation
ADS	25	-	-	+++	Optimal for root initiation
ADS	40	-	-	+++	Enhanced root elongation

Legend: Low reciprocation:+ , Moderate reciprocation:++ , High reciprocation: +++, Very high reciprocation: ++++ and No reciprocation e:- .

Quantitative Estimation of the marker compounds *in-vitro* generated *Morus indica*:

QUERCETIN

For the estimation of Quercetin in the extract, a known amount of dried extract (approx 100mg) was dissolved in methanol and sonicated for 30 minutes to extract Quercitin. The mixture was sieve through a 0.45µm membrane strainer before introducing into HPLC complex under th same condition as quercetin standards. The chromatogram of the extract showed a sharp peak at Rt = 6.8 minutes, matching the retention time of Quercetin standard. The concentration of Quercetin in the extract was determined using the calibration curve. The amount of Quercetin in the extract was determined to approx 23.76 mg/g of extract. This concentration highlights significant concentration of quercitin in the extract, indicating its potential role in contributing its biological activity of the sample.

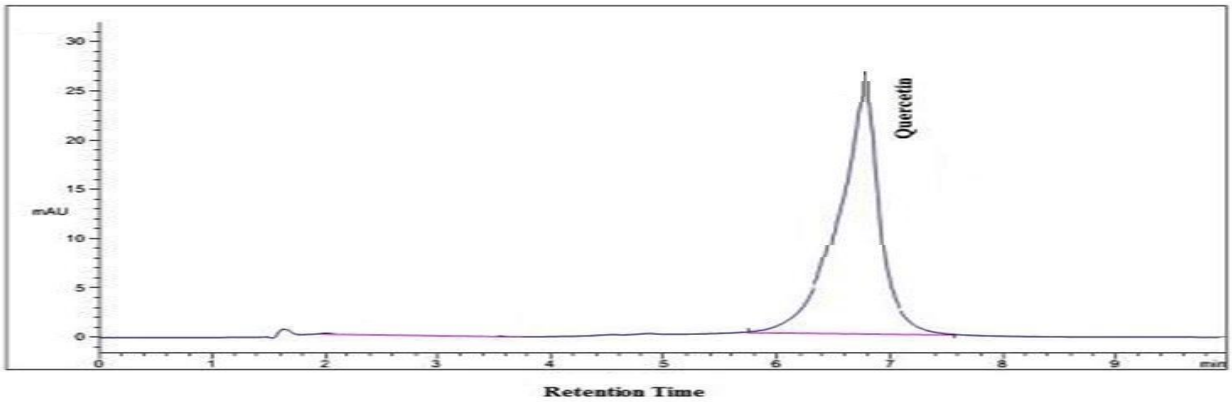


Fig 1 CHROMATOGRAM OF QUERCITIN (SAMPLE)

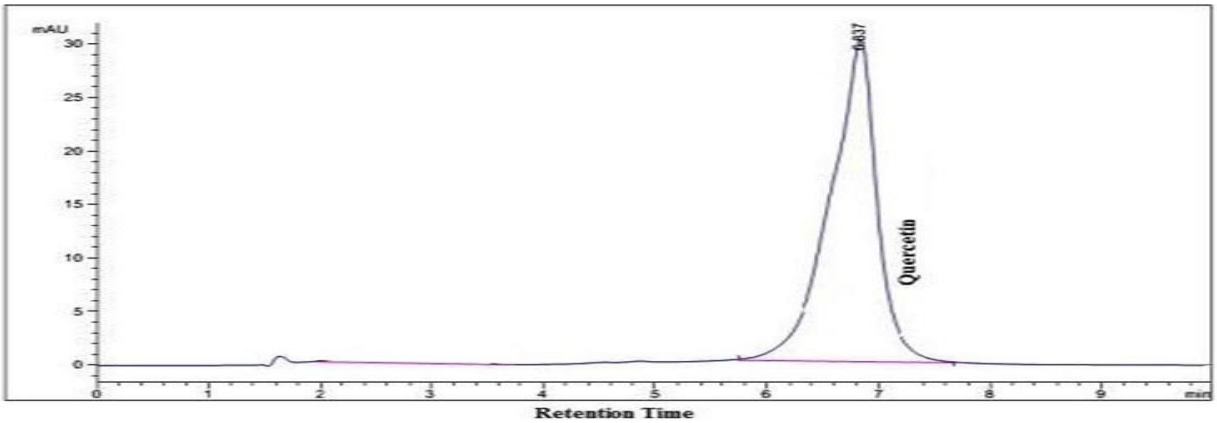


Fig 2. CHROMATOGRAM OF QUERCITIN(STANDARD)

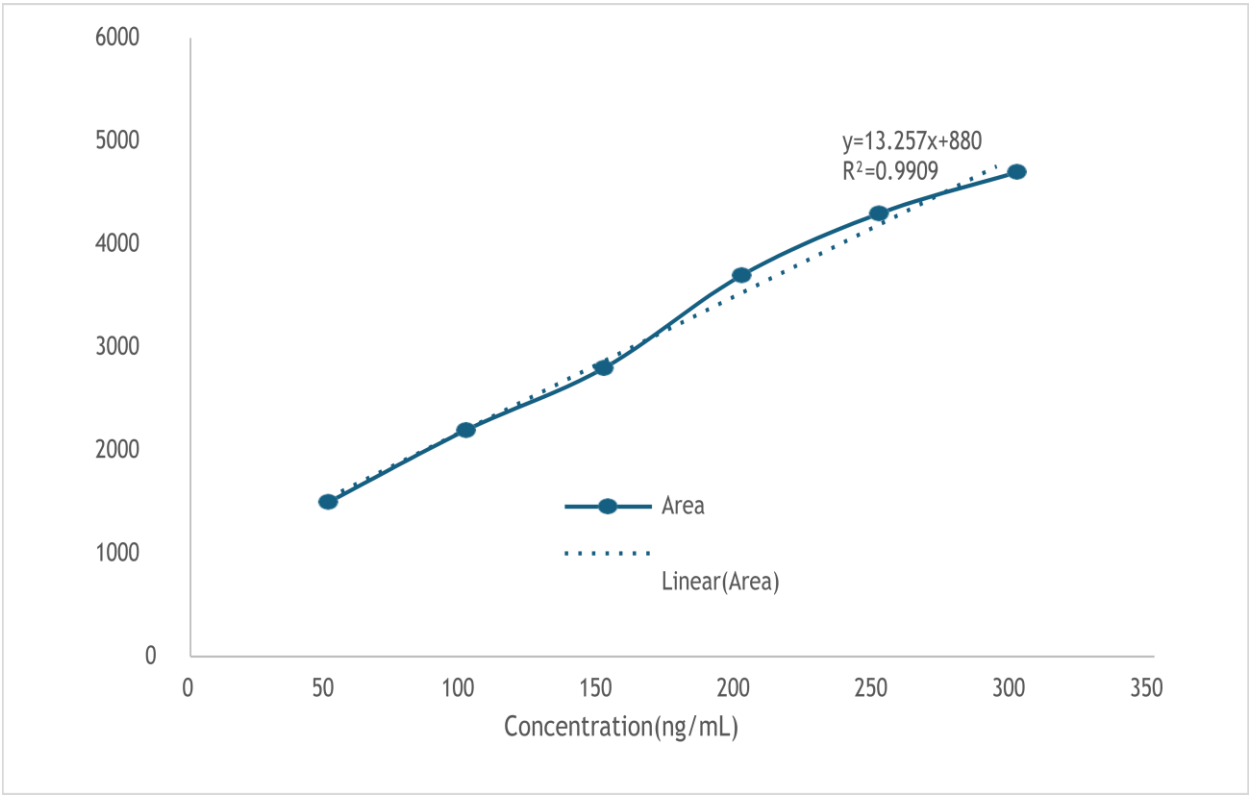


Fig 3. CALIBRATION CURVE OF QUERCITIN

RUTIN

The presence of rutin in the extract was confirmed by a clear peak in chromatogram at Rt=3.3 minutes. The chromatogram exhibit a sharp peak corresponding to rutin with a retention time of 3.3 minutes matching the retention time of rutin standard. Using the calibration

curve the concentration of rutin was determined. The amount of rutin in extract was calculated to be approx 15.91 mg/gm of extract. The concentration confirms the significant presence of rutin in the extract ,highlighting its potential contribution to the biological activity of the extract.

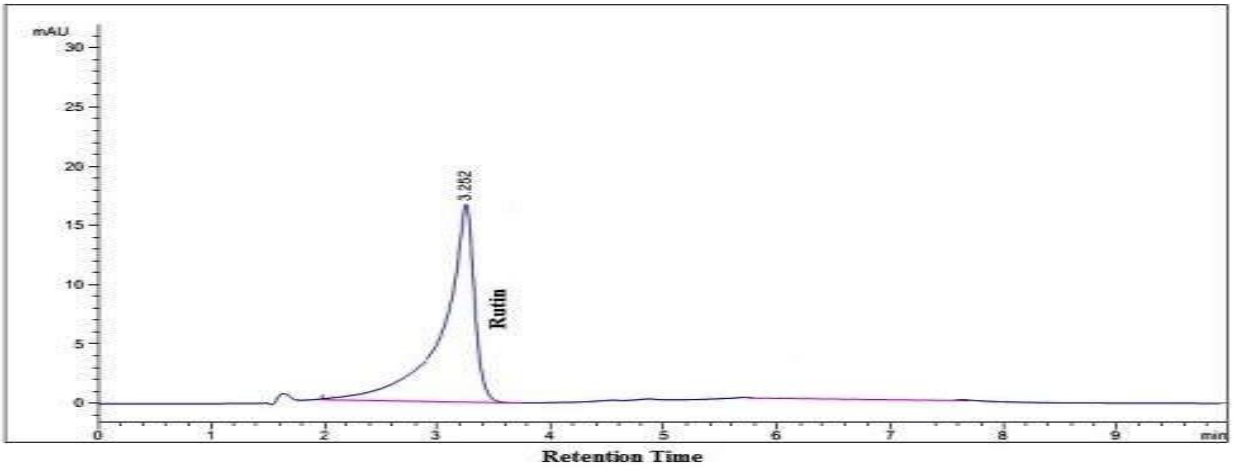


Fig 4. CHROMATOGRAM OF RUTIN(SAMPLE)

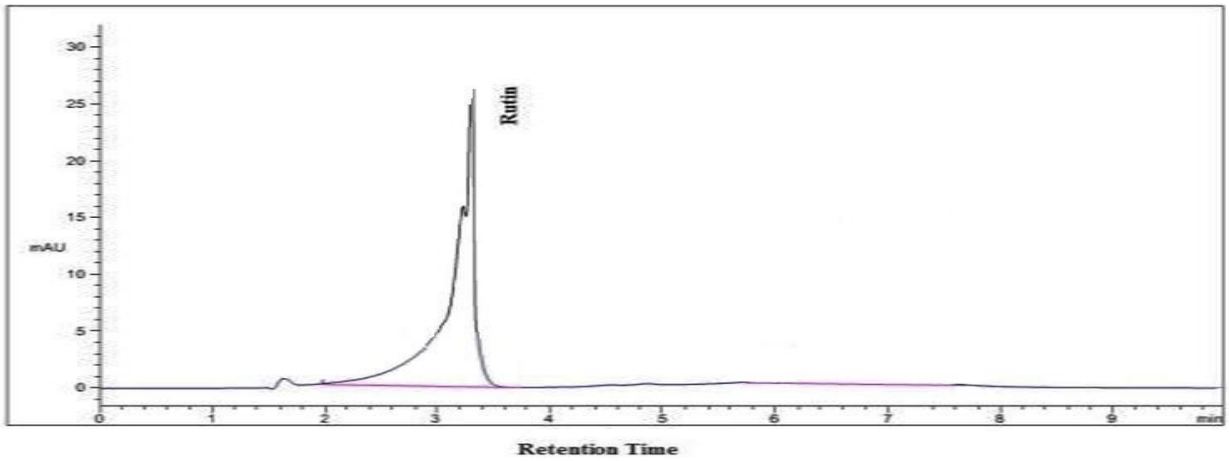


Fig 5. CHROMATOGRAM OF RUTIN(STANDARD)

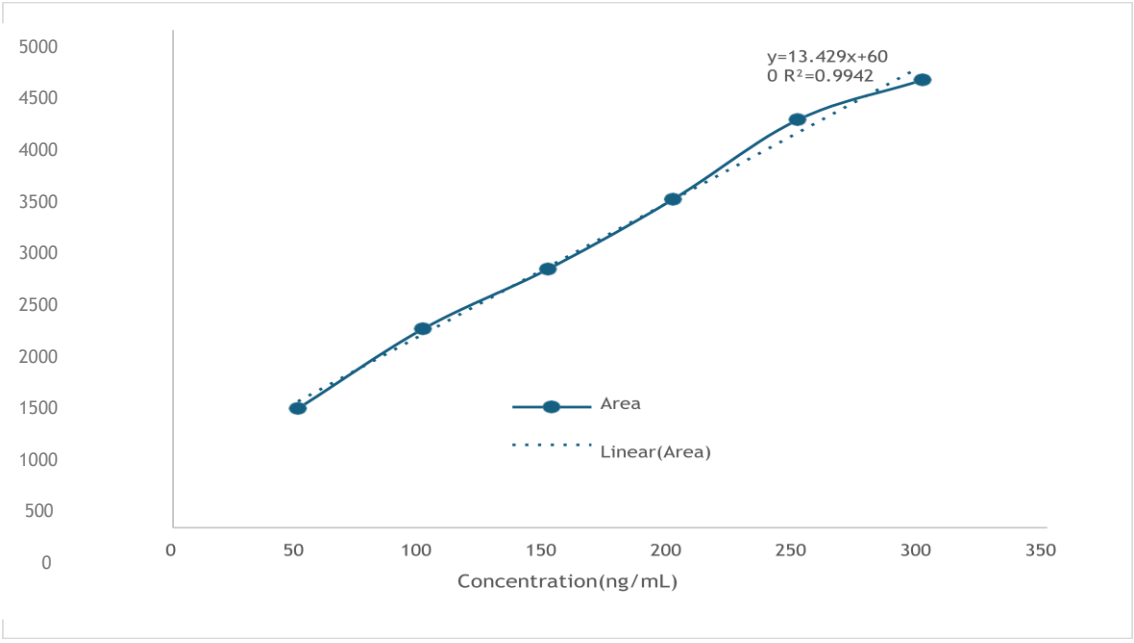


Fig 6. CALIBRATION CURVE OF RUTIN

CHLOROGENIC ACID

A sharp, well defined peak was observed in the chromatograph at the retention time of 25.7 minutes, matching the retention time of chlorogenic acid standard. The peak area was used to observe the amount of chlorogenic acid in the extract by referring to the calibration curve. Based on the area of the peak and calibration curve, the amount of chlorogenic acid in the extract was calculated to be 12.87 mg/g. This concentration highlights the significance presence of chlorogenic acid , indicating its potential role in the biological activity of the extract sample.

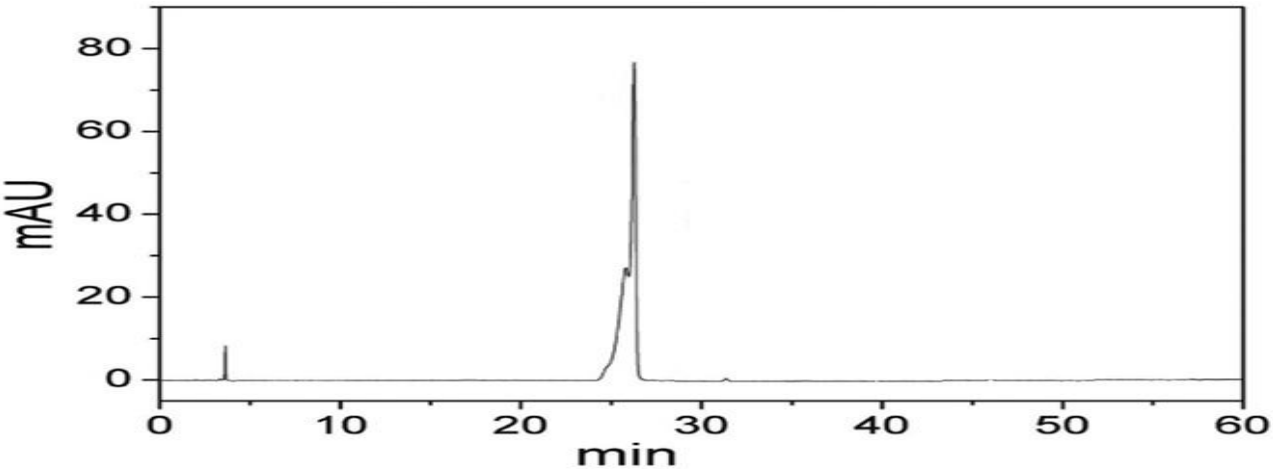


Fig 7. CHROMATOGRAM OF CHLOROGENIC ACID (SAMPLE)

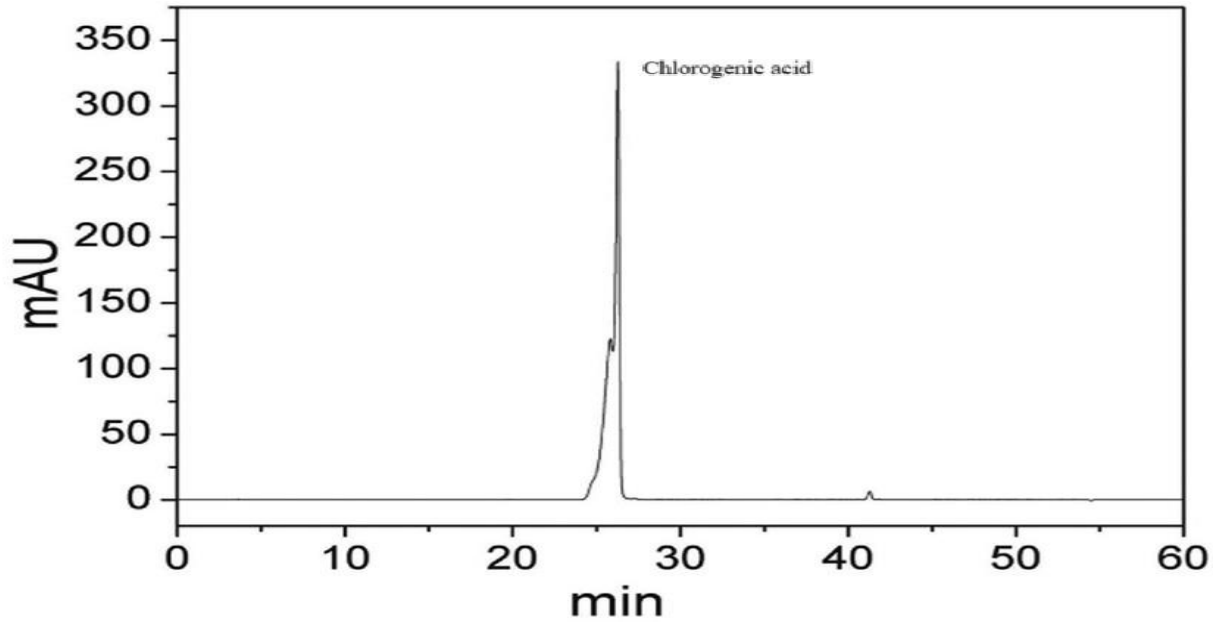


Fig 8. CHROMATOGRAM OF CHLOROGENIC ACID (STANDARD)

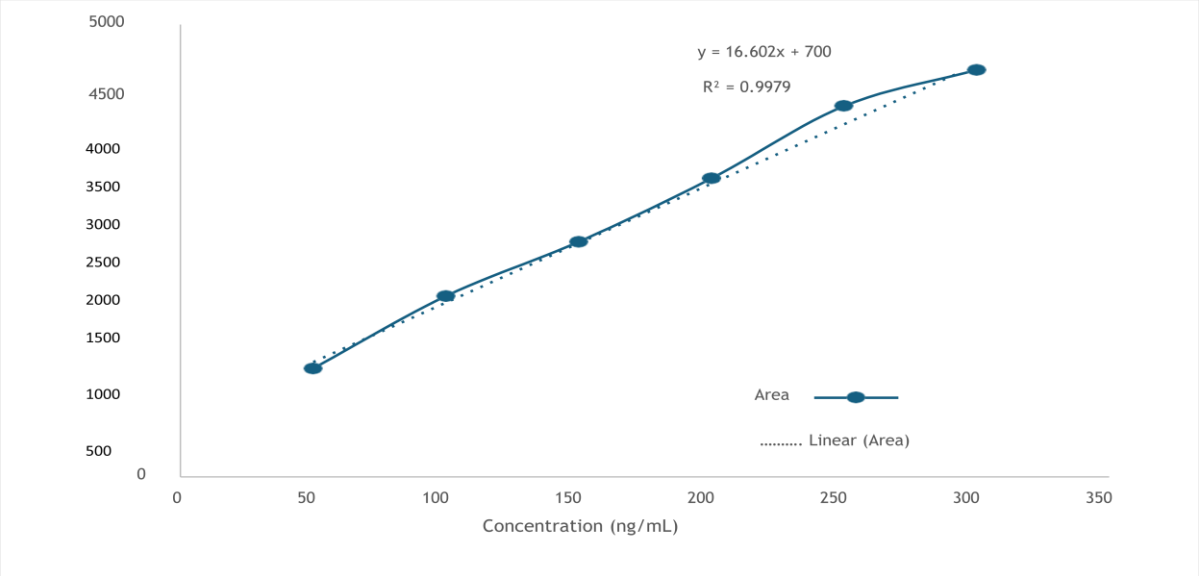


Fig 9. CALIBRATION CURVE OF CHLOROGENIC ACID

CONCLUSION

The technique of *in vitro* multiplication of *Morus indica* has been greatly improved by our work and it provides a great opportunities for commercial propagation. In our findings, growing the *Morus indica* in vitro, kinetin was the only growth regulator which is found to be requires for the proper induction of shoot and tremendous growth. In comparison with the current techniques, using two phase culture method for growing roots, results in decrease culture length and enhanced the quality of roots, which in turn increased plant survivorship. We have established the combined survival and quality management of *Morus indica* in vitro culture by enhanced rooting and improvement. The quantitative estimation of three effective polyphenols were evaluated from the extract of mulberry leaves. These marker compounds can be useful for acclimatization and physiological assessment of mulberry leaves. It is anticipated to have further commercial uses and pave the way for further study on the growth of plant and other quantitative changes of functional constituents having anti-cancer, anti-hypertensive and anti-diabetic activities in *Morus indica*.

DECLERATION OF INTEREST

The authors declare no conflict of interest in this manuscript.

Authors' contributions

This work was carried out in collaboration between all authors. Author NG designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. All Authors managed the analyses of the study, managed the literature searches and All authors read and approved the final manuscript.

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