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RESEARCH ARTICLE

Integrated phytochemical, biological and structure-based *in silico* evaluation of the thyroid modulatory potential of Kaji Nemu (*Citrus limon* (L.) Osbeck) from Assam

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Abstract

Kaji Nemu (*Citrus limon* (L.) Osbeck), a unique citrus from Northeast India with extensive nutritional and therapeutic attributes, has been declared the state fruit of Assam and a geographical indication-certified commodity. Despite its known therapeutic activity, this study highlights the phytochemical potential of the methanolic peel extract of Kaji Nemu and its modulatory effects on thyroid-related pathways. The extraction yield was 10.39 %. Qualitative analysis detected tannins, alkaloids, phenols, saponins, flavonoids, terpenoids and carbohydrates. Quantitative analysis further showed that the extract contained 18.03 mg/g of total phenols, 2.25 mg/g of total flavonoids and 19.52 mg/g of total tannins. The antioxidant activity assay results showed an IC_{50} of 42.00 µg/mL in the DPPH assay, alongside strong FRAP and ABTS scavenging activities. The extract demonstrated cytotoxicity against HEK293 cells in the MTT assay, with an IC_{50} value of 177.7 µg/mL. Gas chromatography-mass spectrometry (GC-MS) analysis identified 93 phytochemicals in the extract and reported antioxidant, anti-inflammatory, anticancer and antithyroid activities. Virtual screening and molecular docking against the thyroid hormone receptor beta 1 (TRβ1) identified 3-tert-Butyl-4-hydroxyanisole, detected through GC-MS analysis, as the lead compound. It showed a strong binding affinity (-6.7 kcal/mol), which was higher than that of the reference antithyroid drug methimazole. HPLC analysis quantified vitamin E and C at 0.09 and 23.97 mg/mL, respectively, supporting its nutritional and functional value. ICP-MS mineral profiling identified sodium as the most abundant element (18.85 mg/L), followed by potassium, calcium and zinc. The study highlights the translational potential of underutilised citrus as safe, novel and effective candidates for thyroid-related treatment, meriting further mechanistic and *in vivo* studies.

Keywords: antioxidant; GC-MS; Kaji Nemu; phytochemical; thyroid hormone receptor β1

Introduction

Thyroid disorder encompasses a diverse set of clinical conditions marked by altered thyroid function and impaired homeostasis of thyroid hormone signalling pathways. Thyroid disease is a highly prevalent endocrine disorder, affecting 5–15 % of the population worldwide. Thyroid disorders are 3–4 times more likely to occur in women than in men. Thyroid hormones regulate various biochemical pathways at the cellular level (1). The most prevalent thyroid disorders include hypothyroidism, characterised by insufficient production of thyroid hormones and hyperthyroidism, which involves excessive hormone production. In addition, thyroid malignancies arise due to genetic and molecular alterations. The majority of thyroid disorders have grown over time due to genetic (mostly mutations), environmental factors, ageing-associated endocrine dysregulation, endocrine-disrupting chemicals, lifestyle factors and most importantly, free radicals (oxidative stress) (2). Importantly, dysregulated thyroid hormone production has been documented to have a pro-oxidant effect, particularly in hyperthyroidism, by enhancing the generation of reactive oxygen species (ROS) through upregulation of cellular energy metabolism and mitochondrial efficiency (1).

The thyroid gland itself requires regulated ROS in the form of H_2O_2 during the biosynthesis of thyroid hormones. Thyroid hormone receptors (TRs), particularly thyroid hormone receptor (TRα and TRβ1), act as nuclear receptors that regulate physiological activities of thyroid hormones within cells. Alterations in these receptors, including mutations or dysregulated expression, may aggravate disease progression by impairing hormone binding and transcriptional activity affecting oxidative pathways in the body (3). The human body works to regulate oxidative stress through various *in vivo* pathways, using antioxidants. Therefore, antioxidants donate electrons and stabilise reactive molecules in body cells. They regarded as free radical neutralisers (4). In recent years, an increase in the demand for the use of exogenous antioxidants in food and medicines as supplementation has been observed and long-term consumption has raised various health-detrimental issues, including the risk of cancer. Moreover, conventional management for thyroid conditions relies on antithyroid drugs together with radioactive iodine therapy and surgical approaches. These treatments are likely to increase the chance of adverse effects, high recurrence rates and lifelong dependency. Interestingly, at the same time, natural antioxidants derived from plant-based formulation contribute a

safer and effective natural alternative to mitigate or reduce the oxidative stress (5). Thus, these 2 targets, TRα and TRβ1, are key molecular elements utilised in the onset and progression of thyroid disorders and offer valuable candidates for structure-based drug discovery.

Medicinal plants are well known for providing phytochemicals that serve as natural antioxidants and offer therapeutic benefits against a variety of human disorders. Since the Ayurvedic period, various phytomedicinal plants have been detailed with regard to therapeutic effect depending on the purity of the raw material (6). It is documented that around 80 % of people on the globe solely depend on medicinal plants and their derivatives to treat the severe health conditions. Among the numerous botanical studies for their remarkable antioxidant property, members of the Rutaceae family, especially citrus plants, have gained considerable attention worldwide. Citrus fruits are considered a reservoir of antioxidants as this plant possesses several essential bioactive compounds, including flavonoids, limonene, hesperidin, essential oil, etc. (6).

India's Northeastern region represents a rich yet largely underexplored source of medicinal plants with potential endocrine relevance. Among fruit crops, citrus species occupy extensive agricultural acreage and are widely distributed across tropical and subtropical regions worldwide. The Indian state Assam is a major region for the cultivation of various citrus species and their varying varieties along with their well-defined odours, appearance, phytochemicals and flavours (7). It has been documented that around 23 species of citrus have been found in the Northeast regions. One such citrus fruit, locally referred to as Kaji Nemu (*Citrus limon* (L.) Osbeck), is an endemic variety grown only in Assam and some regions of Northeastern India. Kaji Nemu or Assam lemon has the uniqueness that it is larger in size, seedless or with very few seeds and has an intense aroma compared to conventional lemon varieties (8). Kaji Nemu plant is a perennial, small, spiny tree that grows 2–3.5 m tall and is characterised by dark green leaves, thorny branches and flowers that are white with purple edges. The fruits are acidic, juicy, elliptic-oval shaped and segmented. Kaji Nemu fruits are available throughout the year; however, their production declines during the November–January period. Due to its monopoly cultivation and indigenous nature, Kaji Nemu was designated a GI-certified product by the Ministry of Commerce and Industry, India, in 2019 and the state fruit of Assam in 2024 (9). It was reported that the Assam lemon has been a chance seedling bred in the citrus station, Burnihut, offspring of the variety Chinakaghi in the village Hashara, district of Sivasagar, Assam. The major producing regions are Dibrugarh, Golaghat, Cachar, Chirang, Nalbari and Dima Hasao (10).

The juice extracted from Kaji Nemu is widely consumed and accounts for approximately 48 % of the total fruit weight. The remaining 52 % consists of peel, seeds and dehydrated residues, which are often discarded as agricultural waste during lime consumption or in the lime juice processing industry. Citrus peels constitute the major portion of this by-product (11). Although generally underutilised in agricultural industries, citrus peels are considered an excellent source of various volatile and non-volatile bioactive compounds. Studies have shown that citrus peels are rich in nutrients and phytochemicals with the potential to mitigate oxidative stress. These compounds may contribute to the management of several health conditions, including inflammation, hypertension, scurvy, respiratory disorders, indigestion, eye infections and skin diseases (4, 11). Recent studies have also reported that citrus peels contain unique flavonoids known as polymethoxyflavones, which are present in relatively high concentrations. These compounds are associated with strong radical scavenging and anti-proliferative activities (12). Extracts of Kaji Nemu have been widely reported to exhibit antitumor, anticancer, anti-inflammatory, antimicrobial and apoptotic properties. However, the therapeutic potential of Kaji Nemu, particularly in the management of thyroid dysfunction associated with oxidative stress, remains inadequately explored.

Although Kaji Nemu is a prominent feature of Assamese cuisine and is used in traditional medicine to treat ailments, there is little scientific evidence for its medicinal efficacy (7). Additionally, since the juice of Kaji Nemu has been extensively studied for its nutritional and medicinal properties, there are few detailed, systematic studies on the methanolic peel extract, especially in the context of Assam lemon (Kaji Nemu), which is rich in unique phytochemicals. Importantly, the therapeutic and clinical relevance of the methanolic peel extract of Kaji Nemu from Assam, relevant in the modulation of thyroid-related disorders, also remains underexplored. This prevailing gap provides a comprehensive research opportunity to investigate its phytochemical characterisation and potential pharmacotherapeutic relevance against thyroid-related complications using modern approaches and to explore its phytochemical composition and potential therapeutic relevance against different health-related conditions using modern approaches. Several preceding scientific studies concerning the anti-thyroid effectiveness reveal the presence of isoflavones and phenolic acid-rich natural substances in citrus, which are frequently eaten as dietary supplements (13). Therefore, in this work, we focused on comprehensive phytochemical profiling, *in vitro* antioxidant analysis, GC-MS and HPLC-based systematic screening for bioactive compounds and nutrient analysis and evaluated their pharmacological potential against thyroid-associated protein targets using advanced *in silico* screening approaches. Moreover, this present study also explored the preliminary cytotoxic potential of the peel extract of Kaji Nemu against the HEK293 cell line, as this cell line can steadily express thyroid-stimulating hormone receptors (TSHR) like TRβ1 under specific conditions. Moreover, few studies have reported that HEK293 cells can be transfected to express thyroid-specific receptors such as TSHR, enabling their use in highly specific reporter gene assays to measure thyroid-stimulating hormone (TSH) activity (14, 15). This cell line has been used as a general research tool for thyroid cancer studies in specific experimental applications such as gene expression studies, drug screening and understanding molecular pathways (14, 15).

This study comprehensively profiles the phytochemical composition and antioxidant potential of Kaji Nemu peel using *in vitro* approaches. It also presents the first *in silico* evaluation of GC-MS identified bioactive compounds from the methanolic peel extract of Kaji Nemu, focusing on their potential modulation of the TRβ1 implicated in thyroid dysfunction. Moreover, cytotoxicity assay against the selected cell line provides a safety profile of the Kaji Nemu peel extract within the biological system, highlighting its therapeutic utility in pharmacological drug discovery. The originality of this research lies in its region-specific focus, specifically the valorisation of the underexplored potential of the peel of Kaji Nemu, which offers novel findings on natural antioxidants, nutraceuticals, cytotoxicity and antithyroid properties that could serve therapeutic,

industrial and pharmacological drug-discovery purposes. Valorisation of citrus peel not only contributes new evidence on its bioactive and nutritional potential but also supports sustainable utilisation of an underexplored part of Kaji Nemu. Using citrus peel promotes sustainability and adds economic value from a readily available fruit-industry byproduct. Therefore, this study sets a pioneering foundation by exploring the untapped therapeutic potential of Kaji Nemu methanolic peel extract against thyroid dysregulation and also elevates the scientific value of a culturally significant, yet underutilised, citrus variety from Assam.

Materials and Methods

Sample collection and authentication

The fresh fruits were collected from the Rajakhat Banekuchi, Nalbari district of Assam, India, in August 2023, which is situated between 26.46–26.51° N latitude and 91.38–91.47° E longitude (Fig. 1). On its southern and eastern sides, the district shares a boundary with Kamrup district. The herbarium was prepared using standard procedures with an accession number GUBH20723 for its identification and authentication, made by the Curator of the Department of Botany, Gauhati University, Assam, India.

Preparation of crude extract

Fresh and good-quality fruits of Kaji Nemu were selected by considering their uniform size, intact peel without physical damage, free of disease and bright green colouration at maturity and were thoroughly rinsed and lightly dried to eliminate excess moisture. The peels were then manually removed aseptically with a sterile knife, dehydrated under shade until the moisture was completely removed and then ground into fine powder in a grinder (16). The powdered sample (30 g) was extracted by cold maceration in 100 % methanol (300 mL) at a 1:10 (w/v) ratio for 72 hr at room temperature. The mixture was stirred magnetically with a polytetrafluoroethylene (PTFE) stir bar for 48 hr. The extract, after agitation, was passed through Whatman No. 1 filter paper and then concentrated via rotary evaporation (Buchi R-124) (16). The resulting extract was maintained in a sterile vessel at 4 °C until additional analysis, referred to as methanolic peel extract (MPECL) of Kaji Nemu.

Estimation of extract yield percentage

After the elimination of the methanol solvent, the methanolic peel extract of Kaji Nemu percentage yield was gained by weighing the dry extract. In the current study, the MPECL yield percentage was obtained following the conventional method. The yield percentage of the MPECL was calculated by employing equation 1 as follows (17).

$$\text{Extraction yield (\%)} = \frac{\text{Weight of crude extract}}{\text{Weight of initial sample}} \times 100 \quad \text{(Eqn. 1)}$$

Preliminary phytochemical profiling

Qualitative screening of phytochemicals

Phytochemicals aimed for preliminary qualitative screening in the MPECL were tannins, alkaloids, saponins, flavonoids, phenols, steroids, terpenoids and glycosides by following published protocols (18). According to the assay's specifications, either the extract solution or the powdered plant material was used.

Test for tannins

Ferric chloride test

1 mL of MPECL and 5 mL of distilled water were combined with just a few traces of 10 % ferric chloride ($FeCl_3$). The bluish-black or brownish-green colour of the solution indicates the detection of tannins.

Lead tetra acetic acid test

0.5 mL of MPECL was added to 1 mL of lead tetra acetate solution; the observed precipitation denotes tannin presence.

Test for alkaloids

Wagner's test

1 mL of filtered MPECL mixed with 1 mL of Wagner's reagent (Iodine in potassium iodide solution). The reddish-brown precipitate in the solution indicates the presence of alkaloids.

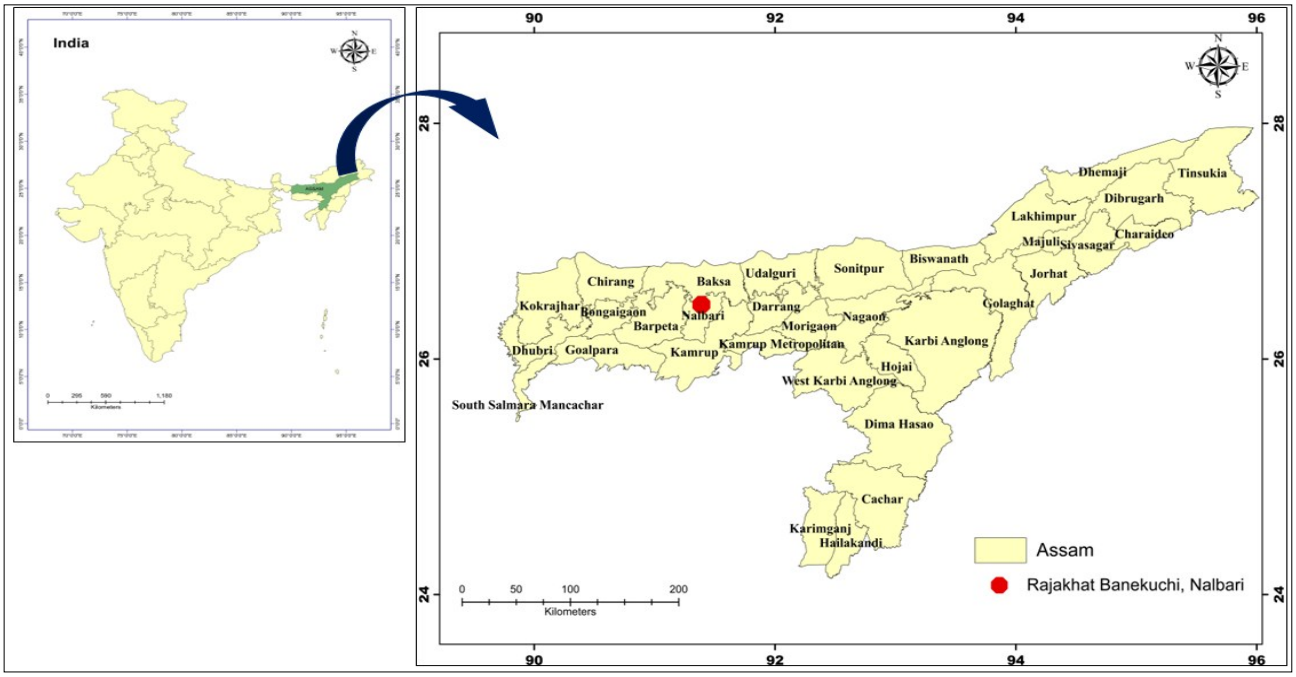

Fig. 1. Location map of study area represent the collection site of Kaji Nemu (*Citrus limon* (L.) Osbeck) (ArcGIS 10.8).

Hager's test

1 mL of the extract sample was combined with 1 mL of Hager's reagent (Saturated solution of picric acid), which showed a yellow colour precipitate in the mixture, signifying the presence of alkaloids.

Test for saponins

Foam test

The appearance of persistent foam upon vigorous shaking of 2 mL MPECL in 10 mL distilled water indicates the presence of saponin.

Kellar-Killiani test

1 mL of MPECL extract mixed with chloroform and allowed to evaporate to dryness, followed by adding 0.4 mL of acetic acid and 0.5 mL of concentrated sulphuric acid, resulting in a blue colour in the solution, signifying saponin.

Test for flavonoids

Alkaline reagent test

1 mL of MPECL was treated with a small drop of 20 % sodium hydroxide (NaOH) and an intense yellow colour was formed in the solution, which decolorised upon the addition of dilute acid, identifying the existence of flavonoids.

Shinoda test

A small piece of magnesium was introduced into the extract, followed by the dropwise addition of concentrated hydrochloric acid. Within several minutes, pink scarlet colour emerged and the presence of flavonoid was depicted.

Test for phenols

Iodine test

Small traces of iodine solution were added to the 2 mL of MPECL and the development of red colour implies the presence of a phenolic compound.

Ferric chloride test

2 mL of MPECL was made and a 10 % solution of $FeCl_3$ was added to it. The presence of a bluish-black colour indicated the presence of phenol.

Test for steroids and terpenoids

Salkowski test

Chloroform and concentrated sulfuric acid (H_2SO_4) were treated with 1 mL of MPECL dropwise and the mixture was agitated. The development of red colour at the bottom of the layer indicates the presence of steroids, whereas the formation of yellow colour in the upper layer indicates the presence of terpenoids.

Liebermann-Burchard test

To the crude extract, a few drops of acetic anhydride were added, followed by heating and cooling. Concentrated sulfuric acid (H_2SO_4) was slowly added down the side of the test tube. A brown ring at the junction of the layers, along with green colour in the upper layer, signifies steroids; deep red colouration implies terpenoids.

Test for carbohydrates

Molisch test

2 mL of MPECL was introduced with 2 mL of Molisch reagent. Subsequently, concentrated H_2SO_4 in small drops was placed along the walls of the test tube. A violet ring formed at the interface between the 2 solutions, indicating that a carbohydrate was present.

Benedict test

Addition of Benedict reagent to the MPECL and boiled in a water bath, the formation of reddish-brown precipitate, which identifies carbohydrate.

Test for glycosides

NaOH test

0.5 mL of the test sample was added to 1 mL of sodium hydroxide (NaOH) solution in a test tube and the solution was mixed gently. The resulting formation of yellow colour in the solution identifies that glycosides were present.

Kellar- Killiani test

1 mL of MPECL extract was mixed with 1 mL of glacial acetic acid and a few drops of $FeCl_3$ before adding H_2SO_4 down the edge of the test tube. When a brown ring is formed at the interface, this is a positive sign of glycoside presence and a violet ring can be formed under the brown ring.

Quantitative phytochemical analysis

Total phenol content (TPC)

The folin-ciocalteu method was employed to estimate the total phenolic content (TPC) of MPECL with the aid of a gallic acid calibration curve with negligible modifications (19). A calibration curve was generated by preparing a series of standard solutions of known (gallic acid) at different concentrations. A stock solution of 1 mg/mL of dried crude extract was prepared in methanol. 200 µL of extract stock solution was mixed with 10 % folin-ciocalteu reagent. Following a 5 min dark incubation, 1.5 mL of 5 % sodium carbonate was introduced, the mixture was vortexed and the final volume was brought to 6 mL. The solution was further incubated in the dark for 2 hr, followed by absorbance measurement. Preparation of standard gallic acid was done in a concentration of 1 mg/mL of methanol in 10 mL volume and further diluted with methanol for working standard concentrations of 25, 50, 100 and 200 µg/mL. Absorbance readings for the standards and sample were obtained at 750 nm using a UV-VIs spectrophotometer. The estimated results were tested in triplicate and presented as gallic acid per mg equivalent (GAE/mg) of extract.

Total flavonoid content (TFC)

Total flavonoid content (TFC) of Kaji Nemu was estimated with the aid of the Quercetin calibration curve with negligible modifications (19). A calibration curve was generated by preparing a series of standard solutions of known flavonoid (Quercetin) at different concentrations (25, 50, 100 and 200 µg/mL). This procedure relies on the amount of yellow-orange colour generation as a result of the reaction of the flavonoid with aluminium chloride ($AlCl_3$) to produce flavonoid- $AlCl_3$ complex. Briefly, 0.25 mg of the dried sample was dissolved in 1.25 mL of distilled water to make sure that there were no impurities that would affect the sensitivity of the spectrophotometric assays and 750 µL of sodium nitrate ($NaNO_2$) was added to it. The solution was mixed and incubated in the dark for 6 min. 150 µL of 10 % $AlCl_3$ was further added to the solution and incubated in the dark for 5 min. Finally, 500 µl of 5 % sodium hydroxide and 275 µL were treated with the mixture and the net volume of 6 mL was achieved by adding methanol. Preparation of standard Quercetin was done in a concentration of 1 mg/mL of methanol in a net volume of 10 mL. This stock was subsequently diluted with methanol to generate working standard solutions of 25, 50, 100 and 200 µg/mL. The absorbance value was

measured for the standard solutions and the sample by using a UV -VIs spectrophotometer at a constant wavelength of 510 nm. The estimated results were tested in triplicate and presented as quercetin per mg equivalent (QE/mg) of extract.

Total tannin content (TTC)

The total tannin content (TTC) of the sample's methanolic extract (1 mg/mL) was assessed spectrophotometrically using a modified procedure (19, 20). 10 % folin reagent and 7.5 % sodium carbonate were mixed together to a combined amount of 3 mL to formulate the reaction mixture. A gallic acid standard curve was utilised for estimating the sample's tannin level. From a stock of 1 mg/mL, multiple quantities of 20, 40, 60, 80 and 100 µg/mL were retrieved to create the standard curve. Three observations of absorbance were performed at a wavelength of 725 nm. 5 g of dried peel powder were obtained employing a soxhlet apparatus with 120 mL of methanol. To disintegrate bioactive compounds, the solvent was repeatedly cycled through the sample 7 or 8 times. By filtering the cooled methanolic extract using a rotary evaporator, which evacuates the solvent with low pressure at 40–50 °C, a concentrated extract was obtained. The after effect residue generated was dried at temperatures between 40 and 50 °C. For subsequent analysis, the dried extract was maintained at 4 °C. The estimated results were tested in triplicate and presented as gallic acid per mg equivalent (GAE/mg) of extract.

In vitro antioxidant activity assays

DPPH free radical scavenging assay

DPPH radical scavenging activity assay was evaluated to confirm the antioxidant capacity of the MPECL by following the protocol with slight variation (21). A 40 µg/mL DPPH solution was prepared in methanol and ascorbic acid was used as the reference antioxidant. Briefly, 4 mg of 2,2-diphenylpicrylhydrazyl (DPPH) (Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in 100 mL of methanol to prepare a stock solution at 40 µg/mL. From the stock solution (1 mg/mL), the working solutions: 10, 150 and 350 µg/mL concentrations were prepared by diluting the DPPH solution with methanol, then incubated in dark conditions for 5 min. The coloured solution was then estimated for absorbance using a UV-VIs spectrophotometer at 517 nm.

A stock solution (1 mg/mL) of the studied sample was also prepared and serial dilutions at 10, 150 and 350 µg/mL in methanol were prepared. 1.5 mL of each diluted sample was dispensed into individual tubes, followed by the addition of 1.5 mL DPPH. The final volume of all the sample tubes was made up to 3 mL of methanol. After that, all the sample tubes were incubated for 5 min in dark conditions at room temperature. Finally, the absorbance was estimated at 517 nm by using a UV-VIs spectrophotometer. The percentage inhibition or scavenging activity was calculated using equation 2.

$$\text{Scavenging activity (\%)} = \frac{\text{Abs (Control)} - \text{Abs (sample)}}{\text{Abs (Control)}} \times 100 \quad \text{(Eqn. 2)}$$

IC_{50} values were evaluated from the inhibition (%) versus concentration sigmoidal curve, via non-linear regression analysis.

Ferric reducing antioxidant power (FRAP) assay

FRAP reagent was made by combining 300 mM acetate buffer (pH = 3.6), 10mM TPTZ solution in 40 mM HCL and 20 mM ferric chloride in water with a ratio of 10:1:1(v/v/v) and incubated in a water bath at 37 °C for 15 min before testing activity. 100 µL of sample extract was solubilised in 1 mL of methanol and 3 mL of FRAP reagent and kept for 5 min at 37 °C and allowed to react for 4–5 min. After incubation, the increased absorbance was observed at 593 nm by the use of spectrophotometer. By the aid of variable concentrations: 0.1, 0.2, 0.4, 0.6 and 0.8 µM, the standard curve of $FeSO_4$ was established and the FRAP values were quantified by relating the absorbance change of the test mixture to a ferrous sulfate standard curve. The acquired FRAP value was in the form of mM of Fe equivalents per mL of sample. The absorbance was recorded at 519 nm (22).

ABTS assay

Considering the technique outlined by, the antioxidant efficacy of the MPECL against ABTS was tested with slight modifications (23). A stock solution for the ABTS test comprised 0.18 g of ABTS in 50 mL of distilled water. The functional solution was developed by diluting the ABTS solution with methanol to get an absorbance of approximately 0.70 ± 0.02 at 745 nm, utilising the spectrophotometer. Ascorbic acid serves as a standard, at a concentration of 0.44 mg/mL. 3 mL of this diluted ABTS solution were used to add to various sample concentrations (0, 2, 5, 7 and 10 µg/mL). Following 15 min of dark, room temperature incubation, the solutions were evaluated for absorbance at 745 nm by using equation 3.

$$\text{Scavenging activity (\%)} = \frac{\text{Abs (Control)} - \text{Abs (sample)}}{\text{Abs (Control)}} \times 100 \quad \text{(Eqn. 3)}$$

The antioxidant strength of the MPECL was mentioned as EC_{50} or scavenging percentage of ABTS anti-radical activity (22).

Cytotoxicity assay of MPECL

HEK293 cell culture

Human embryonic kidney cell line (HEK293) employed in the present study was acquired from NCCS, Pune. The cell cultures were propagated in minimum essential medium (MEM) containing 10 % fetal bovine serum, 1 % sodium bicarbonate, 1 % non-essential amino acids and 1 % sodium pyruvate. cells were maintained at 37 °C in a humidified 5 % CO_2 atmosphere for further use (24).

Cell viability analysis

Through the MTT test, the effect of the MPECL on cell viability was investigated. The soluble MTT salt, "3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide", that embodies the standard role of mitochondrial dehydrogenase performance and cell viability, is metabolically transformed into an insoluble coloured formazan end product, which is determined spectrophotometrically. The number of live cells is directly proportionately measured by the level of activity of mitochondrial dehydrogenase in living cells (24).

The effective cytotoxic concentration of the sample drug was determined using the MTT assay. A 96-well microtiter plate was seeded with 1×10^4 HEK293 cells in each well. Subsequently, the cells were incubated for 24 hr. Subsequently, a crude extract of the sample was put in at a concentration of 100–1000 µg/mL and the cells were allowed to culture for an additional 24 hr. Every well was then inoculated with 100 µL of complete growth medium containing 0.5mg/mL MTT. The cells were propagated for a duration of 5 hr, the media used was cautiously disposed of and the formed formazan

crystals were submerged in 100 µL DMSO for 30 min. The IC_{50} value was subsequently determined by measuring absorbance at 570 and 650 nm using a microplate reader. Equation 4 mentioned below was implemented for estimating the percentage of cell viability.

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of treated cells - Background absorbance (b)}}{\text{Absorbance of untreated (c) - Background absorbance (b)}} \times 100 \quad \text{(Eqn. 4)}$$

Where, b = blank and c = control

GC-MS analysis of MPECL

The GC-MS analysis was performed in a mass spectrometer system with a split/splitless injection port in electron impact mode. The GC column of the system used was a 5 MS capillary column combined with silica of 30 m × 0.25 mm and 0.25 µm film thickness specification. The temperature for the run was initially set at an isothermal phase of 60 °C for 3 min, then shot straightaway up to 230 °C with a rise of 5 °C/min. Injector and transfer line temperatures were operated at 290 and 230°C, respectively. The outlet of the GC column was attached to the ion source block. Ultrapure helium was employed as carrier gas, which was allowed to flow at 1 mL/min and the sample load volume was set at 0.2 µL peak area percentage was calculated automatically by chromeleon™ software (25). Qualitative chromatographic analysis was carried out in certain strengths of prepared dilute solutions. Compound identification was achieved by correlating experimentally obtained retention index and mass spectrum to NIST library-provided authentic standards.

ADMET analysis

The bioactive molecules revealed by GC-MS analysis were taken from the PubChem database of NCBI (https://www.ncbi.nlm.nih.gov). The OpenBabel tool was executed to retrieve the compounds in SDF format. Protox 3.0 was deployed for assessing hepatotoxicity, nephrotoxicity, cardiotoxicity, mutagenicity, immunogenicity and cytotoxicity of the compounds. The SwissADME tool was utilised to evaluate the lead likeliness, pharmacokinetic characteristics and violations of drug ability rules, including Lipinnski, Ghose and Veber (26).

Virtual screening

A total of 29 ADMET-passed drug-like compounds were subjected to virtual screening identified through GC-MS against TRβ1. The 3D crystal structure of TRβ1 was accessed from the protein data bank (PDB ID: 1NAX). Structure-based virtual screening was performed using AutoDock Vina, which employs an efficient scoring function to predict binding strength and molecular interactions between ligands and the target protein. Ligands were modelled flexibly and the protein was treated as rigid during the docking process. Compounds were ranked based on their binding energy scores (kcal/mol) and those exhibiting higher binding affinity than the reference antithyroid drug methimazole were shortlisted for further evaluation. Preparation of protein includes elimination of co-crystallised ligands and water molecules, adding polar hydrogen atoms and assignment of Gasteiger charges using AutoDock tools (v1.5.6) (26).

Molecular docking

The PDB database facilitated 3D structure files of the targeted protein, namely TRβ1 (PDB ID: 1NAX), was subsequently modified utilising the Pymol software (26). AutoDock Vina was used to conduct protein -ligand docking interactions. The ligands were maintained flexible, whereas the protein was maintained rigid. The protein and ligands were furnished with Gasteiger charges and polar hydrogen. The GL software package's AutoDock tools (v.1.5.6) was employed for generating PDBQT files characterising the proteins and ligands. The protein-ligand docking complexes were evaluated by the Lamarckian genetic algorithm (LGA) method. UCSF Chimaera version 1.14 was adopted for evaluating the binding interactions of the docking complexes (26).

Vitamin E and C analysis

Vitamin E analysis

Vitamin E content in the extract was determined using high-performance liquid chromatography (HPLC) equipped with an Agilent TC-C18(2) column (4.6 × 250 mm, 5 µm). The mobile phase consisted of methanol and acetonitrile (30:70, v/v), which was filtered and degassed before use. The flow rate was maintained at 1.0 mL/min, with detection at 295 nm and an injection volume of 10 µL at ambient temperature.

A standard stock solution of tocopherol (1 mg/mL) was prepared in HPLC-grade water and further diluted to obtain working standards of 50, 25 and 12.5 µg/mL for calibration curve preparation. For sample preparation, 1 mg of the extract was dissolved in 1 mL of HPLC-grade water, sonicated for 15 min, diluted as required and filtered through a 0.22 µm nylon membrane filter before injection. Vitamin E concentration in the sample was determined by comparing chromatographic peak areas with the standard calibration curve (27, 28).

Vitamin C analysis

Vitamin C content in the peel extract of Kaji Nemu was determined using the same HPLC conditions described for Vitamin E analysis, with the exception that the mobile phase consisted of methanol and water (30:70, v/v). A standard stock solution of vitamin C (1 mg/mL) was prepared in HPLC-grade water and subsequently diluted to obtain working standards of 100, 50, 25 and 12.5 µg/mL for calibration curve preparation. For sample preparation, 100 µL of the extract was diluted with distilled water, sonicated for 15 min and filtered through a 0.22 µm nylon membrane filter before injection into the HPLC system. The concentration of vitamin C in the extract was determined by comparing the chromatographic peak areas with those obtained from the standard calibration curve (27, 28).

Mineral analysis

Sodium (Na), potassium (K), calcium (Ca), zinc (Zn), magnesium (Mg) and manganese (Mn) concentrations were evaluated by employing a nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) digestion procedure. Briefly, 1 g of fine powder test sample was taken into a testing tube, followed by treatment with a mixture of nitric acid and perchloric (Merck KGaA, Darmstadt, Germany) in a 5:1 v/v ratio. This mixture was then put on the stove at a temperature of 90 °C and the temperature kept rising in every 2 min by 10 °C until it attained the temperature of 170 °C. At this temperature, a few drops of hydrogen peroxide were added to the solution and a white fume formed. The resulting mixture was allowed to cool and deionised water was added subsequently. Finally, the mixture was filtered

through Whatman no. 42 filter paper. The solutions obtained were subjected to inductively coupled plasma quadrupole mass spectrometry to determine heavy metals (29).

Statistical analysis

All experiments in the present study were carried out in triplicate (n = 3) and the obtained data were expressed as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism version 10.4.2 (GraphPad Software Inc., USA) and Microsoft Excel. Differences between the treated and control groups were evaluated for statistical significance. The significance level was adopted for all the assessments, $p<0.05$. Each value is significantly justified using a one-sample T-test with two-tailed distributions for antioxidant assay, Duncan's multiple range test (DMRT) for mineral analysis and linear regression analysis and the goodness of fit was assessed using the coefficient of determination (R^2) for TPC, TFC, TTC and vitamin analysis.

Results

Percentage extraction yield of MPECL

The percentage yield of MPECL was found to be the maximum percentage yield -10.39 %. The sample appeared as a dark brown powdery solid form.

Qualitative preliminary phytochemical analysis

The studied sample acquired by the methanolic peel extract of Kaji Nemu was employed in the qualitative phytochemical profiling. From the heat map (Fig. 2), peel extract phenols, saponins and flavonoids showed a high level of presence. Similarly, tannin, carbohydrate, alkaloid and glycosides were found to be in moderate presence in both tests. However, existence of steroids and terpenoids in the salkowksi test was found to be low where in Liebermaann-Burchard test was found to be intense presence in the extract.

Quantitative analysis of TPC, TFC and TTC

The analysis was conducted to determine the specific amount of phytocomponents present in the MPECL. The results revealed that TPC in MPECL was found to be more 18.03 ± 0.006 mg/g GAE equivalent of extract. The standard curve of gallic acid was found as $y = 0.019x$, $R^2 = 0.997$ (Fig. 3A), showing a monotonic increase of absorbance with increasing concentration. On the other hand, Flavonoids are another class of polyphenolic antioxidant phytochemicals that were recorded to be 2.25 ± 0.003 mg/g quercetin equivalent of extract in MPECL. The quercetin calibration curve with regression coefficient value for TFC was estimated as $y = 0.0011x$, $R^2 = 0.9897$ (Fig. 3B), also showed a linear increase of absorbance with increasing concentration. Similarly, total tannin content was revealed to be 19.52 ± 0.208 mg/g gallic acid equivalent of extract MPECL. The standard curve of gallic acid was found as $y = 0.0048x$, $R^2 = 0.976$, with a linear increase of absorbance with increasing concentration (Fig. 3C).

In vitro antioxidant activity assay

Natural antioxidants become essential for their effective health benefits with added nutritive value in the diet due to their lack of toxicity and carcinogenic activity. The radical quenching activity of methanolic peel extract was determined through DPPH, FRAP and ABTS activity assays, because they have free radical scavenging potential (21, 22).

The antioxidant strength assessment by the DPPH assay was presented in the form of an IC_{50} value. Results reported that an MPECL showed significant antioxidant activity with a lower IC_{50} value of 42.00194 ± 0.15 µg/mL relative to the standard ascorbic acid with an IC_{50} value of 95.8459 ± 0.18 µg/mL. The increasing scavenging activity with increasing concentration of the sample and standard was displayed in Fig. 4A. According to the finding, MPECL extract showed significantly different DPPH radical activity compared to the standard antioxidant (ascorbic acid) with $p<0.05$ (Fig. 4A).

MPECL demonstrated significant ABTS radical scavenging activity. The radical's inhibition (%) efficiency was found to depend on the concentration. At the concentration of 50 µg/mL, the MPECL exhibited inhibition values 19.835 % while the standard ascorbic acid showed 60.92 %. The EC_{50} of peel extract was found to be a moderate value of 36.79 ± 0.03 µg/mL, while that of ascorbic acid was found to be a lower value of 4.651 ± 0.19 µg/mL. Higher EC_{50} value means the extract required a higher concentration to attained 50 % inhibition in the assay. As MPECL showed significantly different scavenging activity compared to the standard with $p<0.05$, highlighting its potent antioxidant potential (Fig. 4B).

Additionally, from the result of the FRAP assay in MPECL, the EC_{50} for the MPECL was recorded as 0.5116 ± 0.005 mM $FeSO_4$ equivalent/mL. The extract showed increased antioxidant activity in a dose-dependent manner. The curve for the standard drug ferrous sulphate with R^2 value 0.9972, $y = 0.3536$, was displayed in Fig 5A. While in the MPECL, the R^2 value was found to be 0.9853, indicating a strong relation of extract concentration with the radical inhibition (Fig. 5B). The present findings reported that MPECL exhibited a much lower EC_{50} value, denoting its excellent antioxidant potency in the assay.

MTTS assay

The cytotoxicity effect on the HEK293 cells by a range of crude extract concentrations (Fig. 6) was observed. The IC_{50} value was observed at 177.7 µg/mL concentration of crude methanolic peel extract. Cell viability decreased with increasing extract dose. Cell viability (%) of the target cell line was diminished significantly under the treatment with crude extract at the concentration ranges from 200–400 µg/mL in MTT assay. A significant difference was detected between the treated and untreated (Control) cells during the experiment. A marked dose-dependent decrease in cell viability was detected at concentrations 200 µg/mL and above, with viability falling below 50 %, indicating cytotoxic effects. The curve for cell viability against concentration and representative bright field images of the cells in control and cells treated with 100, 200 and 300 µg/mL concentration of crude extract are shown in Fig. 6.

GC-MS analysis

GC-MS was done to find out the specific phytocompounds and percentage composition in MPECL. Current GC-MS screening reported the presence of a total of 93 compounds, among which some were also derivatives found in the MPECL. The chromatogram of GC-MS analysis was displayed in Fig. 7 and the phytoconstituents with their respective retention time (RT), peak area (%), molecular formula and their biological classes of each compound were presented in Table 1. From GC-MS profiling, a total of 14 % phytochemical composition of the peel extract of Kaji Nemu was identified under the classes of sesquiterpenoids and terpenoids, respectively, where the sesquiterpenoid class compounds showed versatile diversity in composition, such as sesquiterpenoid alcohol, ester and lactone. While 13 % phytochemical constituents were

Table 1. Different phytocomponents of methanolic peel extract of *Citrus limon* identified by GC-MS analysis are shown with their respective molecular weight, molecular formula, retention time and peak area percentage

Peak No.	RT (min)	Peak area (%)	Compound Name	Biological class	Molecular formula
			Bicyclo[3.1.1] hept-2-ene, 2,6-dimethyl-6-(4 - methyl-3-pentenyl)	Sesquiterpenoid	$C_{15}H_{24}$
1	7.91	3.48	1,3,6,10-Dodecatetraene, 3,7,11-trimethyl-, (Z, E	Sesquiterpenoid	$C_{15}H_{24}$
			cis-a-Bergamotene	Sesquiterpenoid	$C_{15}H_{24}$
			Cyclopentane, 1-acetyl-1,2-epoxy-	Terpenoids	$C_7H_{10}O_2$
2	4.735	0.06	1H-Azonine, octahydro-1-nitroso-	Alkaloid	$C_8H_{16}N_2O$
			2,3-Dimethylfumaric acid	Fumaric acid (organic acid)	$C_6H_8O_4$
			4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	Polyphenol	$C_6H_8O_4$
3	5.351	3.19	2-Propyl-tetrahydropyran-3-ol	Terpenoid	$C_8H_{16}O_2$
			2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	Furanone	$C_6H_8O_4$
			Benzofuran, 2,3-dihydro-	Coumarin	C_8H_8O
4	5.929	2.81	6-Methylenebicyclo [3.2.0] hept-3-en-2-one	Terpenoid	C_8H_8O
			Benzene, (ethenyloxy)-	Phenol	C_8H_8O
			2-Methoxy-4-vinylphenol	Phenol	$C_9H_{10}O_2$
5	6.759	0.24	Phenol, 5-ethenyl-2-methoxy-	Phenol	$C_9H_{10}O_2$
			4-Hydroxy-2-methylacetophenone	Phenol	$C_9H_{10}O$
			d-Mannose	Carbohydrate	$C_6H_{12}O_6$
6	8.065	2.79	Desulphosinigrin	Glucosinolate	$C_{10}H_{17}NO_6S$
			d-Glycero-d-ido-heptose	Carbohydrate	$C_7H_{14}O7$
			7-epi-cis-sesquisabinene hydrate	Sesquiterpenoids	$C_{15}H_{26}O$
7	8.653	0.49	7-epi-trans-sesquisabinene hydrate	Sesquiterpenoids	$C_{15}H_{26}O$
			cis-a-Bisabolene	Sesquiterpene	$C_{15}H_{24}$
			Phenol, 4-ethenyl-2,6-dimethoxy	Phenol	$C_{10}H_{14}O_3$
8	9.286	0.7	3-tert-Butyl-4-hydroxyanisole	Phenol	$C_{11}H_{16}O_2$
			2,5-Dihydroxy-4-isopropyl-2,4,6-cycloheptatrien-1-one	Hydroxytropone	$C_{10}H_{12}O_3$
			11-Oxatetracyclo [5.3.2.0(2,7).0(2,8)] dodecan-9-one	Sesquiterpene lactone	$C_{12}H_{18}O$
9	10.35	29.73	6-Methoxychroman-2-one	Coumarins	$C_{10}H_{10}O_3$
			3,9-Epoxytricyclo [4.2.1.1(2,4)] decan-10-one, 9-methyl	Epoxides	$C_{11}H_{12}O_2$
			d-Mannose	Carbohydrate	$C_6H_{12}O_6$
10	11.292	0.35	Desulphosinigrin	Glucosinolate	$C_{10}H_{17}NO_6S$
			Melezitose	Carbohydrate	$C_{18}H_{32}O_{16}$
			ß-D-Glucopyranose, 4-O-ß-D-galactopyranosy	Carbohydrate	$C_{12}H_{22}O_{11}$
11	12.054	1.42	d-Mannose	Carbohydrate	$C_6H_{12}O_6$
			Melezitose	Carbohydrate	$C_{18}H_{32}O_{16}$
			17-Octadecynoic acid	Lipid	$C_{18}H_{32}O_2$
12	13.03	0.06	Ethanol, 2-(9-octadecenyloxy)-, (Z)	Lipid	$C_{20}H_{40}O_2$
			9-Tetradecen-1-ol, acetate, (E)	Lipid	$C_{16}H_{30}O_2$
			6,10-Dimethoxy-3,3-dimethyl-1-methylsulfanyl-2-aza-spiro [4.5]de	Spiro [4.5] decane	$C_{14}H_{19}NO_3S$
13	13.66	1.41	N-(7-Amino-3-hydroxy-2,3-dihydro-1-benzofuran-5-yl) acetamide	Benzofuran	$C_{22}H_{18}N_2O_5S$
			trans-Sinapaldehyde	Cinnamaldehyde	$C_{11}H_{12}O_4$
			Hexadecanoic acid, methyl ester	Fatty acid methyl ester	$C_{17}H_{34}O_2$
14	14.404	1.37	Pentadecanoic acid, 14-methyl-, methyl ester	Fatty acid methyl ester	$C_{17}H_{34}O_2$
			Tetradecanoic acid, 12-methyl-, methyl ester	Fatty acid methyl ester	$C_{16}H_{32}O_2$
			n-Hexadecanoic acid	Fatty acid	$C_{16}H_{32}O_2$
15	15.074	9.63	Pentadecanoic acid	Fatty acid	$C_{16}H_{32}O_2$
			l-(+)-Ascorbic acid 2,6-dihexadecanoate	L-ascorbic acid	$C_{38}H_{68}O_8$
16	15.625	21.71	1H-Tetrazole, 5-(3,4-dimethoxyphenyl)	Tetrazole	$C_9H_{10}N_4O_2$
			2H-1-Benzopyran-2-one, 4,7-dimethoxy	Coumarins	$C_{11}H_{10}O_4$
			i-Propyl 15-methylhexadecanoate	Fatty acid ester	$C_{20}H_{40}O_2$
17	16.619	0.24	Heptadecanoic acid	Fatty acid	$C_{17}H_{34}O_2$
			n-Hexadecanoic acid	Fatty acid	$C_{16}H_{32}O_2$
			11-Octadecenoic acid, methyl ester	Fatty acid methyl ester	$C_{19}H_{36}O_2$
18	17.312	2.05	10-Octadecenoic acid, methyl ester	Fatty acid methyl ester	$C_{19}H_{36}O_2$
			trans-13-Octadecenoic acid, methyl ester	Fatty acid methyl esters	$C_{19}H_{36}O_2$
			Oleic Acid	Fatty acid	$C_{18}H_{34}O_2$
19	17.993	15.3	cis-Vaccenic acid	Omega-7 fatty acids.	$C_{18}H_{34}O_2$
			2-Methyl-Z, Z-3,13-octadecadieno	Terpenoid	$C_{19}H_{36}O$
			cis-Adamantane-2-carboxylic acid, 4-hydroxy	Adamantane	$C_{11}H_{16}O_3$
20	20.914	0.16	3-Buten-2-amine, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl	Sesquiterpenoid	$C_{13}H_{20}O$
			2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	Cinnamic acid,	$C_{18}H_{26}O_3$

			ß-D-Mannofuranoside, farnesy	Terpenoids	$C_{21}H_{36}O_6$
21	21.649	0.61	Formic acid, 3,7,11-trimethyl-1,6,10-dodecatrien-3-yl ester	Sesquiterpene alcohol	$C_{16}H_{26}O_2$
			ß-d-Mannofuranoside, O-gerany	Glycoloipid	$C_{17}H_{32}O_6$
			Cholestan-3-ol, 2-methylene-, (3ß,5a)	Steroid	$C_{28}H_{48}O$
22	22.329	0.02	1-Heptatriacotanol	Fatty alcohols	$C_{37}H_{76}O$
			Formic acid, 3,7,11-trimethyl-1,6,10-dodecatrien-3-yl este	Sesquiterpenoid ester	$C_{16}H_{26}O_2$
			Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	Lipid	$C_{19}H_{38}O_4$
23	23.788	0.14	Glycerol 1-palmitate	Lipid	$C_{19}H_{38}O_4$
			Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	Lipid	$C_{35}H_{68}O_5$
			Phthalic acid, di(2-propylpentyl) ester	Phthalic acid esters	$C_{24}H_{38}O_4$
24	24.411	0.35	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	Phthalic acid esters	$C_{24}H_{38}O_4$
			Phthalic acid, di(oct-3-yl) ester	Phthalic acid esters	$C_{24}H_{38}O_4$
			Pregnan-20-one, 3,17-dihydroxy-, (3ß,5ß)	Steroid	$C_{21}H_{34}O_3$
25	26.414	0.54	6-Hydroxy-powelline-N-nitroso-7-demethoxy-, aldehyde	Alkaloid.	$C_{17}H_{14}O_5$
			4ß-Methylandrostane2,3-diol-1,17-dione	Steroid	$C_{20}H_{30}O_4$
			9-(2-Hydroxy-3-methyl-3-butenyloxy)-4-methoxyfuro(3,2-g)chro	Furanocoumarin	$C17H_{16}O_6$
26	27.203	0.74	(R)-9-(2,3-Dihydroxy-3-methylbutoxy)-4-methoxy-7H-furo(3,2-g)	Furanocoumarin	$C_{17}H_{18}O_7$
			(R)-9-((3,3-Dimethyl-2-oxiranyl) methoxy)-4-methoxyfuro(3,2-g) c	Coumarin	$C_{17}H_{16}O_6$
			(R)-9-(2,3-Dihydroxy-3-methylbutoxy)-4-methoxy-7H-furo(3,2-g)	Furocoumarins	$C_{17}H_{18}O_7$
27	28.907	1.03	9-(2-Hydroxy-3-methyl-3-butenyloxy)-4-methoxyfuro(3,2-g)chro	Furanocoumarin	$C_{17}H_{16}O_6$
			(R)-9-((3,3-Dimethyl-2-oxiranyl) methoxy)-4-methoxyfuro(3,2-g) c	Furanocoumarin	$C_{17}H_{16}O_6$
			a-Tocopheryl acetate	Vitamin E	$C_{31}H_{52}O_3$
28	31.927	0.57	dl-a-Tocopherol	Vitamin E	$C_{29}H_{50}O_2$
			Vitamin E	Vitamin E	$C_{29}H_{50}O_2$
			W-18	Opioid	$C_{19}H_{20}ClN_3O_4S$
29	33.355	0.12	Glycine, N-[(3a,5ß)-24-oxo-3-[(trimethylsilyl)oxy] cholan-24-yl]-,	Steroid	$C_{36}H_{69}NO_6Si_3$
			Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl	Siloxane	$C_{12}H_{36}O_5Si_6$
			Sitosterol	Phytosterols	$C_{29}H_{50}O$
30	34.066	0.46	ß-Sitosterol	Phytosterols	$C_{29}H_{50}O$
			17-(1,5-Dimethylhexyl)-10,13-dimethyl-4-vinylhexadecahydrocyc	Steroid	$C_{29}H_{50}O$
			Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecam	Organosilicon	$C_{16}H_{48}O_7Si_8$
31	35.593	0.11	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl	Organosilicon	$C_{12}H_{36}O_5Si_6$
			Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethy	Siloxane	$C_{14}H_{42}O_6Si_7$

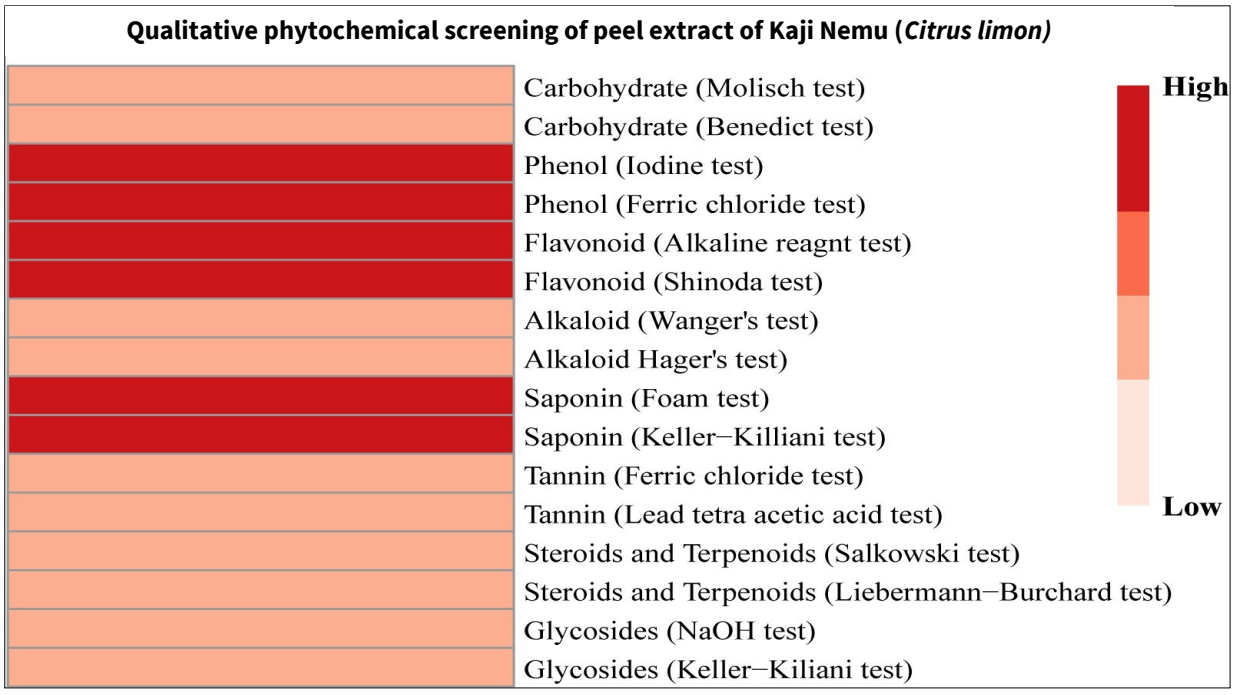

Fig. 2. Heat map depicting the qualitative phytochemical profile of the methanolic peel extract of *Citrus limon*. Colour intensity represents qualitative levels of the phytochemical presence (High and Low) as determined by standard biochemical tests.

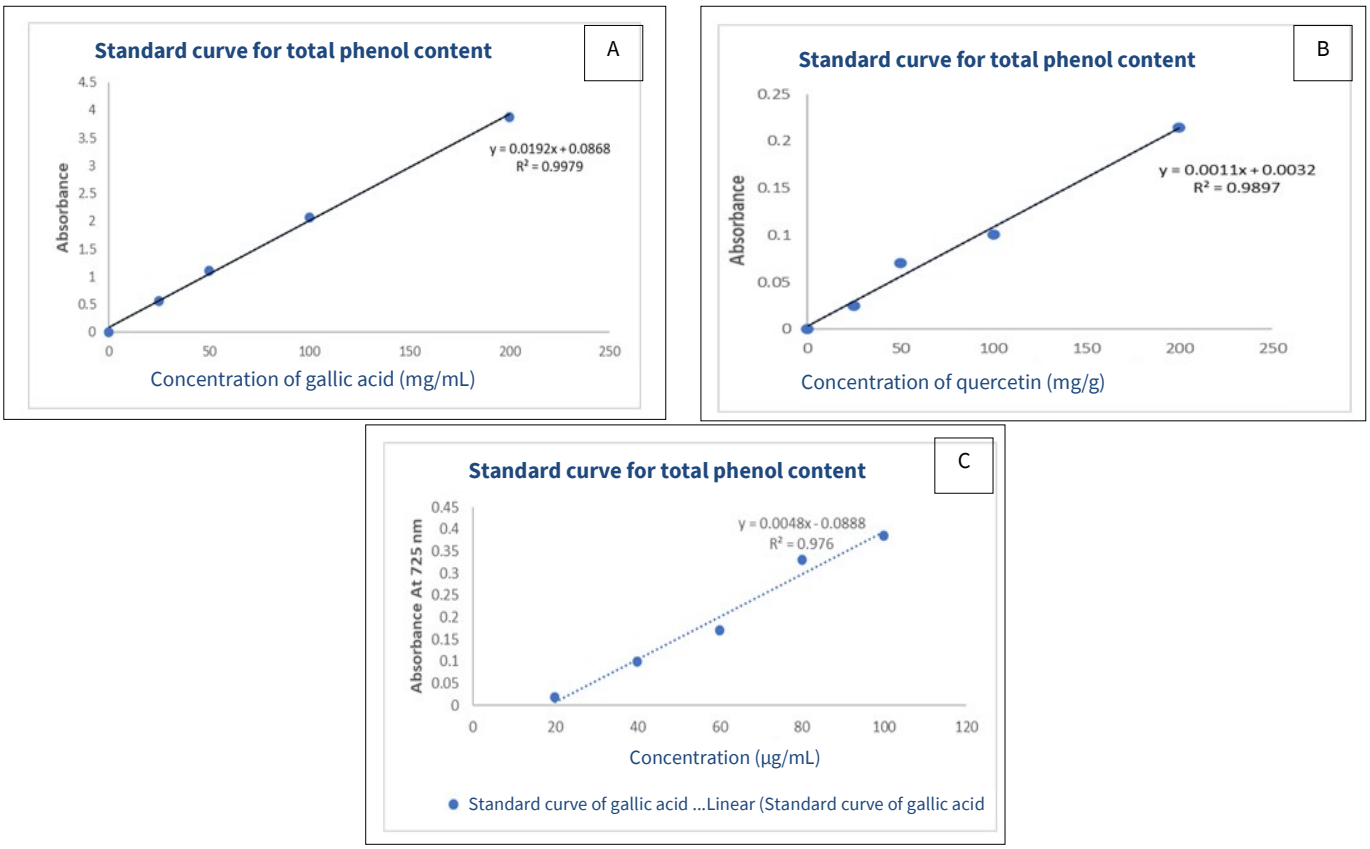

Fig. 3. The standard calibration curve of: A. Gallic acid for total phenolic content; B. Quercetin calibration curve for total flavonoid content; C. Gallic acid for total tannin content.

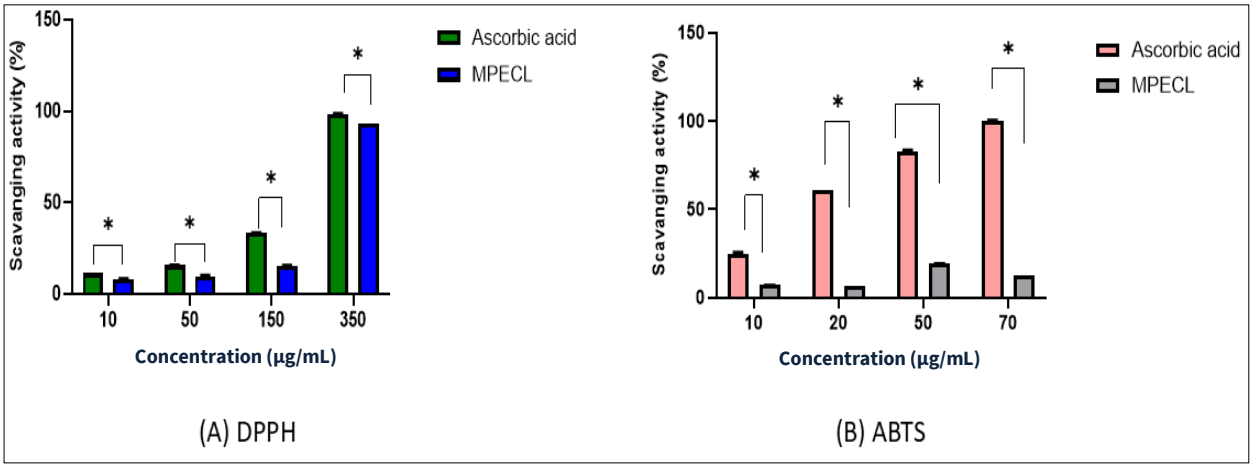

Fig. 4. Bar Graphs showing antioxidant activity assay of methanolic peel extract of Kaji Nemu using: A. DPPH method; B. ABTS assay. Each value is significantly justified using one sample T-test with two tailed distributions, values are expressed mean ± SD, $p<0.05$ (n=3).

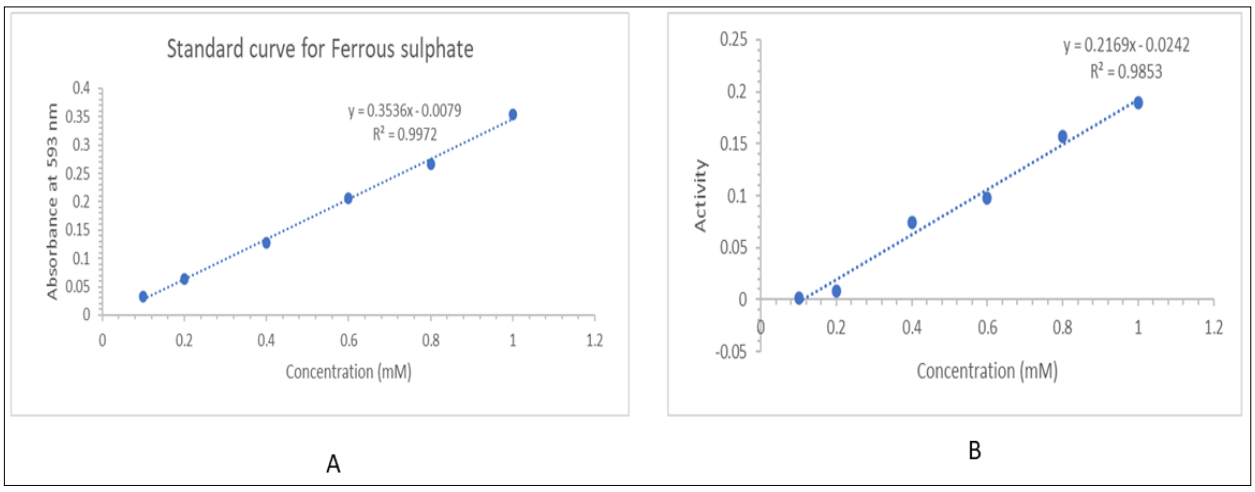

Fig. 5. Graph showing: A. standard curve for ferrous sulphate; B. antioxidant activity of the extract for FRAP assay.

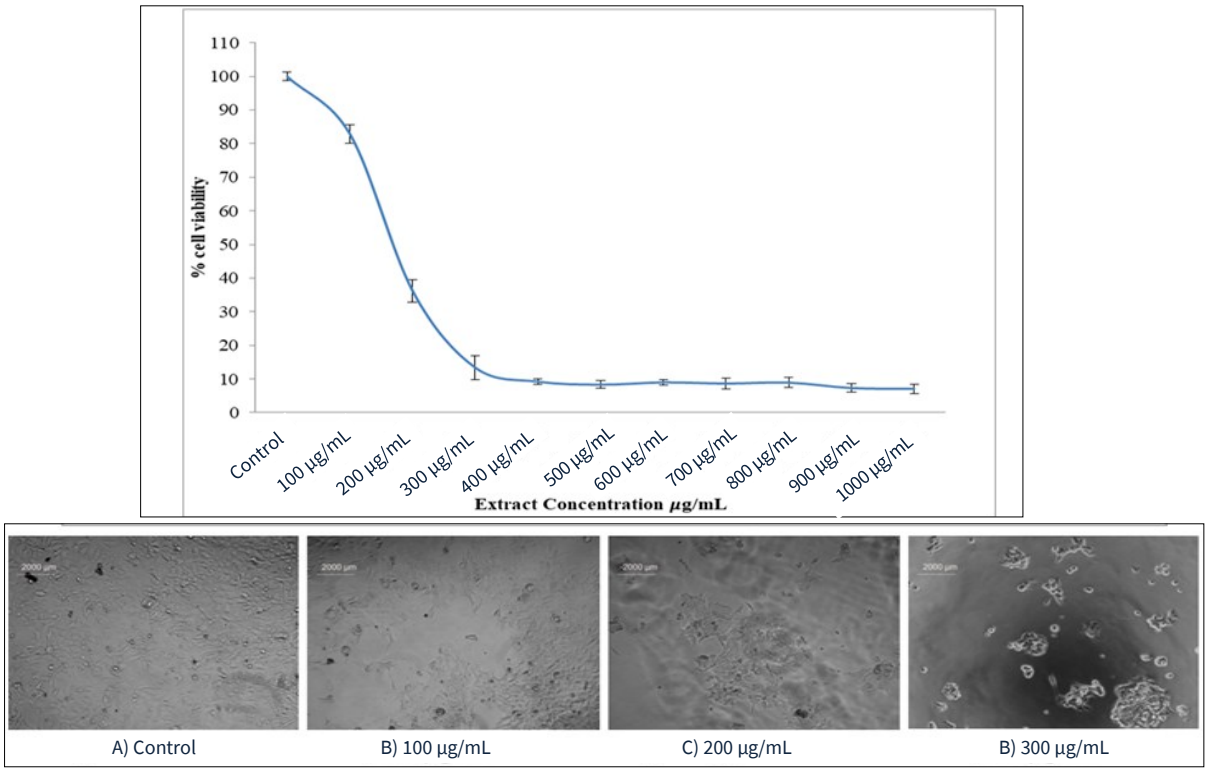

Fig. 6. Graph showing cell viability (%) at different concentration ranges from 100–1000 µg/mL. Bright field images represent cytotoxicity effects on the HEK293 cell line under the treatment of crude methanolic peel extract of *Citrus limon*. A. Control; B. cell viability of 100 µg/mL of MPECL; C. cell viability of 200 µg/mL of MPECL; D. cell viability of 300 µg/mL of MPECL.

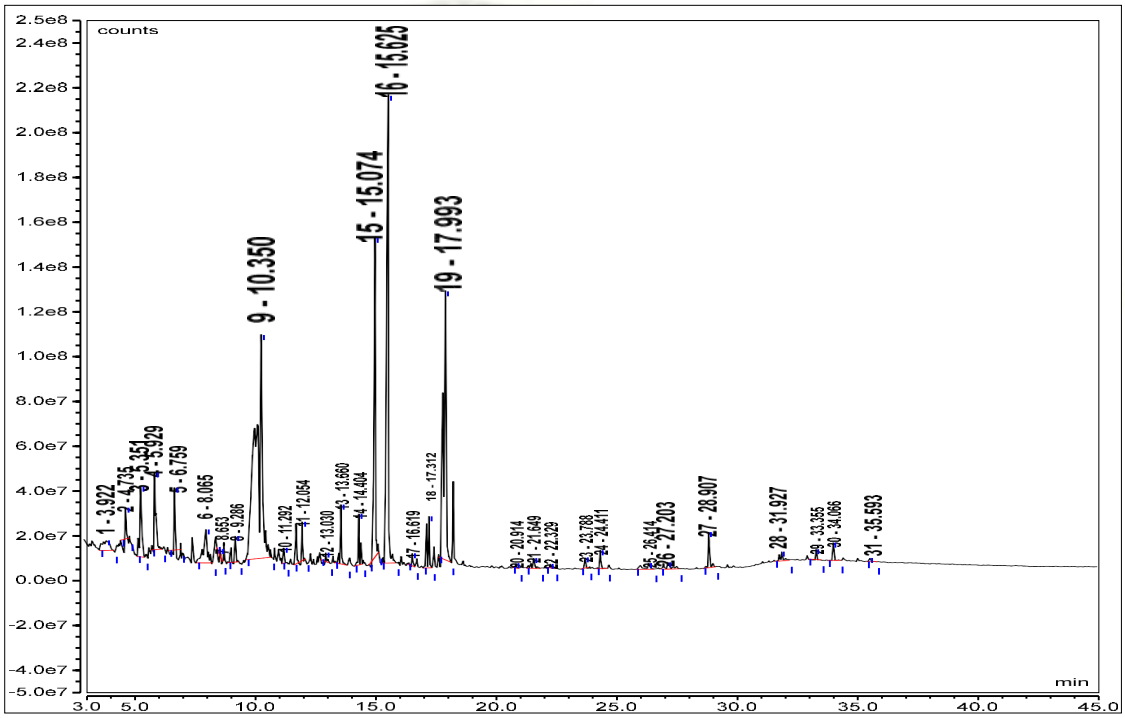

Fig. 7. Chromatogram of GC-MS analysis of methanolic peel extract of *Citrus limon.*

identified as fatty acids and derivatives such as fatty acid esters and methyl esters respectively, followed by 9 % compound identified as coumarin and its derivatives, 7 and 8 % phytochemical composition were identified as phenol and carbohydrate respectively. The rest of the phytochemicals were identified as alkaloids, steroids, epoxides, cinnamic acid in very less number.

Moreover, GC-MS screening results reported that 11-Oxatetracyclo [5.3.2.0(2,7).0(2,8)] dodecan-9-one (Sesquiterpene lactone), 6-Methoxychroman-2-one (Coumarin) and 3,9-Epoxytricyclo [4.2.1.1(2,4)] decan-10-one, 9-methyl (Epoxides) displayed highest peak area of 29.73 % with RT 10.350. Similarly, 2H-1-Benzopyran-2-one,5,7-dimethoxy (Coumarin), 1H-Tetrazole, 5-(3,4-dimethoxyphenyl) (Tetrazole) also displayed a high peak area of 21.75 % with RT 15.62. Moreover, oleic acid (Fatty acid), cis-vaccenic acid (omega-7 fatty acids) and 2-methyl-Z, Z-3,13-octadecadieno (Terpenoid) exhibited a high peak area of 15.30 % with RT 17.993. n-Hexadecenoic acid, pentadecanoic acid (Fatty acid) and l-(+)-ascorbic acid 2,6-dihexadecanoate (L-ascorbic acid) also displayed a high peak area of 9.63 % with RT 15.04.

Virtual screening and molecular docking analysis

Virtual screening was conducted to identify potential bioactive constituents from the GC-MS-identified phytochemicals of MPECL that

could effectively interact with TRβ1. The structural details of all the 31 compounds were characterised through GC-MS screening (Table 1) and were obtained from the PubChem compound database of NCBI (https://www.ncbi.nlm.nih.gov/) and subjected to physicochemical and ADMET evaluation. Based on drug-likeness criteria, toxicity profiling and rule-based filtering (Lipinski, Ghose and Veber rules), 29 compounds were shortlisted for binding affinity and molecular docking studies. An in-depth explanation of the physicochemical attributes of the 29 compounds was included in the Supplementary Table. Molecular docking was carried out using AutoDock Vina in PyRx 8.0. to evaluate the binding behaviour and interaction profiles of the chosen ligands with TRβ1 (PDB ID: 1NAX). The docking results revealed variable binding energies among the screened compounds, indicating differential binding efficiencies toward the active site of the receptor. Out of the evaluated 29 compounds, one lead compound, namely 3-tert-Butyl-4-hydroxyanisole with a molecular weight of 180.24 g/mol received consideration pursuant to binding affinity, drug likeness rule violations and toxicity evaluations. Further, in support of the molecular docking analysis, it became apparent that the compound had lowest binding energy along with minimal toxicity and no rule violations. The binding energy of 3-tert-Butyl-4-hydroxyanisole with TRβ1 (1NAX) was found to be -6.7 kcal/mol. Comparatively, the reference antithyroid drug methimazole (PubChem ID: 1349907) with TRβ1 (1NAX) showed a lower binding affinity toward TRβ1 with a binding energy of -4.0 kcal/mol, indicating a weaker interaction than the identified lead compound (26). The superior docking score of 3-tert-Butyl-4-hydroxyanisole highlights its potential as a promising modulator of TRβ1 activity. Further reinforcing the finding of molecular docking, our prior investigation on LC-MS derived three high-score lead candidates showed higher binding affinity across the three thyroid target proteins thyroid peroxidase (TPO), TRα1 and TRβ1, exceeding the reference methimazole in binding affinity. This suggests Kaji Nemu peel may be a potent modulator against thyroid-related disorders.

Interaction analysis revealed that 3-tert-Butyl-4-hydroxyanisole formed key stabilising interactions within the ligand-binding domain of TRβ1, including hydrogen bonding and hydrophobic interactions with crucial amino acid residues involved in receptor activation and regulation. The observed interactions substantially contribute to the stability and selectivity of the protein-ligand complex. Both 3D and 2D interactions in the figure illustrate the binding modes of each ligand within the binding pockets of the target protein, highlighting key interacting residues and overall interaction profiles. A remarkable π-alkyl and alkyl interactions with hydrophobic moieties LEU330, MET313 and ALA297, which may promote a snug binding conformation and significant van der Waals stabilisation. A conventional hydrogen bond is ASN331, strengthening the binding of the ligand by polar interaction. The 2D interaction in the diagram further supports these results by highlighting several π-alkyl interactions, which imply the ligand binds within a mainly hydrophobic pocket and utilises its aromatic groups to enhance binding affinity (Fig. 8). In contrast, the control ligand methimazole displays greater polarity and a less extensive interaction network. The 3D model identifies three hydrogen bonds with THR329, ASN331 and ARG316 that are important for orienting the ligand, though they may not confer the wider stabilising interactions exhibited by the candidate compound. In a 2D structure, a conventional hydrogen bond was formed with Tyr32; there was also the presence of non-bonded interaction seen in the form of a carbon-hydrogen bond given by Leu360, Gly357 and Ala318 (Fig. 9A and 9B). Despite forming strong polar contacts, methimazole shows restricted engagement in the pocket due to the absence of broad hydrophobic interactions. This may underlie the lower overall binding stability with moderate inhibition.

Toxicity profiling

In silico toxicity profiling of the GC-MS-identified phytochemicals from the methanolic peel extract of Kaji Nemu (MPECL) was performed to assess their safety and pharmacological feasibility. A total of 29 shortlisted compounds, selected after physicochemical and drug-likeness screening, were subjected to toxicity prediction using the ProTox-3.0 platform. The evaluated toxicity endpoints included hepatotoxicity, nephrotoxicity, cardiotoxicity, mutagenicity, immunotoxicity and cytotoxicity. Among the screened compounds, 3-tert-Butyl-4-hydroxyanisole (CSID8456) was predicted to be inactive for hepatotoxic, nephrotoxic, cardiotoxic, mutagenic and immunotoxic effects, indicating minimal toxic risk (Table 2). Additionally, this suggests that compounds exhibited no toxicity risk across the organ system.

Table 2. Organ toxicity and toxicological endpoints of the predicted CID8456 compound using the ProTox server

Classification	Targets	Prediction of CID8456
Organ toxicity	Hepatotoxicity	Inactive
Organ toxicity	Nephrotoxicity	Inactive
Organ toxicity	Cardiotoxicity	Inactive
Toxicity end points	Immunotoxicity	Inactive
Toxicity end points	Mutagenicity	Inactive
Toxicity end points	Cytotoxicity	Inactive

Vitamin E and C analysis

The concentration of Vitamin E and C in MPECL appeared to be 0.93 and 23.79 mg/mL, respectively (Table 3). The chromatogram of both Vitamin C and E is displayed in Fig. 10A and 10B, respectively. The calibration curve of standard tocopherol and ascorbic acid for both Vitamin E and Vitamin C against the area under the peak eluted at a retention time (RT) of 18.509 and 2.180 min, respectively, demonstrated a satisfactory linearity with a correlation coefficient (R^2) of 0.982 and 0.9969, respectively, within the concentration range.

Table 3. Showing the concentration of Vitamin E and Vitamin C of Methanolic peel extract of *Citrus limon* using the HPLC method with their respective peak area and retention time

Vitamin	Retention time (min)	Peak area (%)	Concentration (mg/mL)
E	18.28	346	0.09
C	3.09	389023	2.374

Mineral analysis

Among the estimated minerals, Na was found highest in concentration at 18.85 mg/L, followed by K with 6.93 mg/L, Ca was 5.7 mg/L and Zn was 4.1 mg/L, respectively, recorded in the peel extract. All the elements exhibited significant differences in concentration, with p <0.001 were displayed in Fig. 11.

Discussion

There is an increasing focus in scientific research on the prompt evaluation of oxidative stress or inadequate antioxidant capacity and its detrimental effects on human physiology, including various life-threatening endocrine disorders. Therefore, the existence of diverse phytochemicals in plants holds promising pharmacological

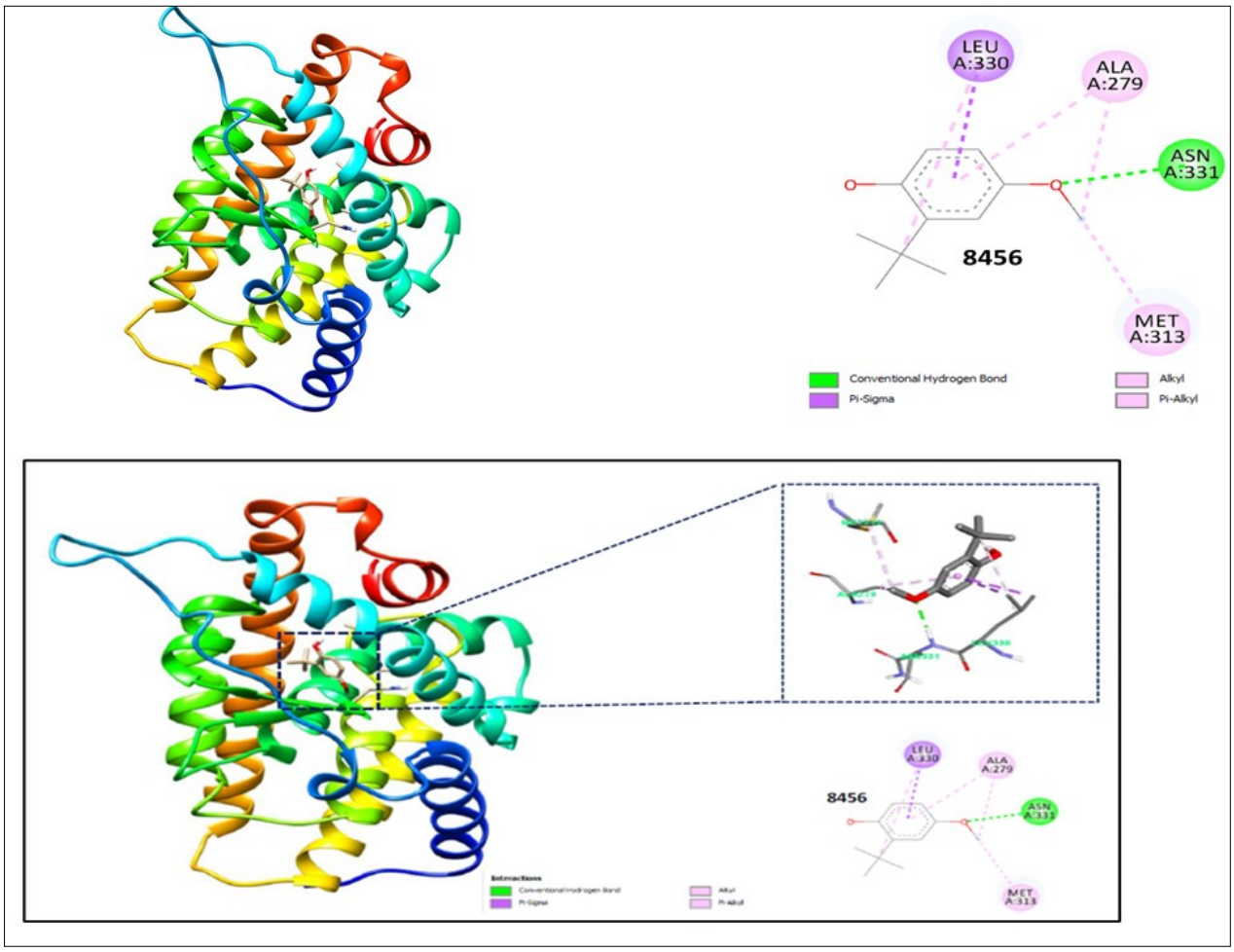

Fig. 8. 3D and 2D structure of 1NAX with 3-tert-butyl-4-hydroxyanisole (CID-8456) docked complex.

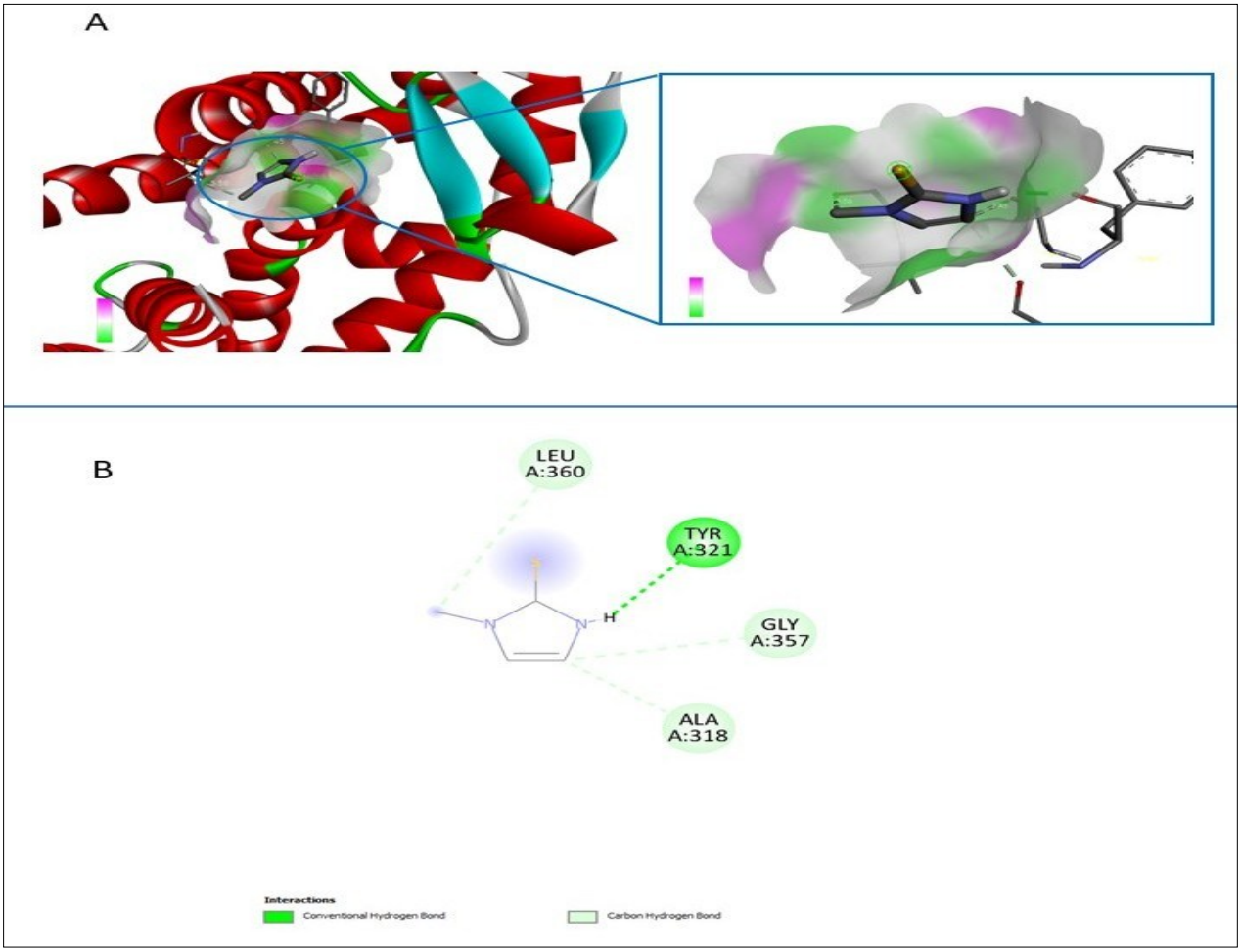

Fig. 9. 3D and 2D structure of 1NAX protein with reference compound (CID-1349907) docked complex.

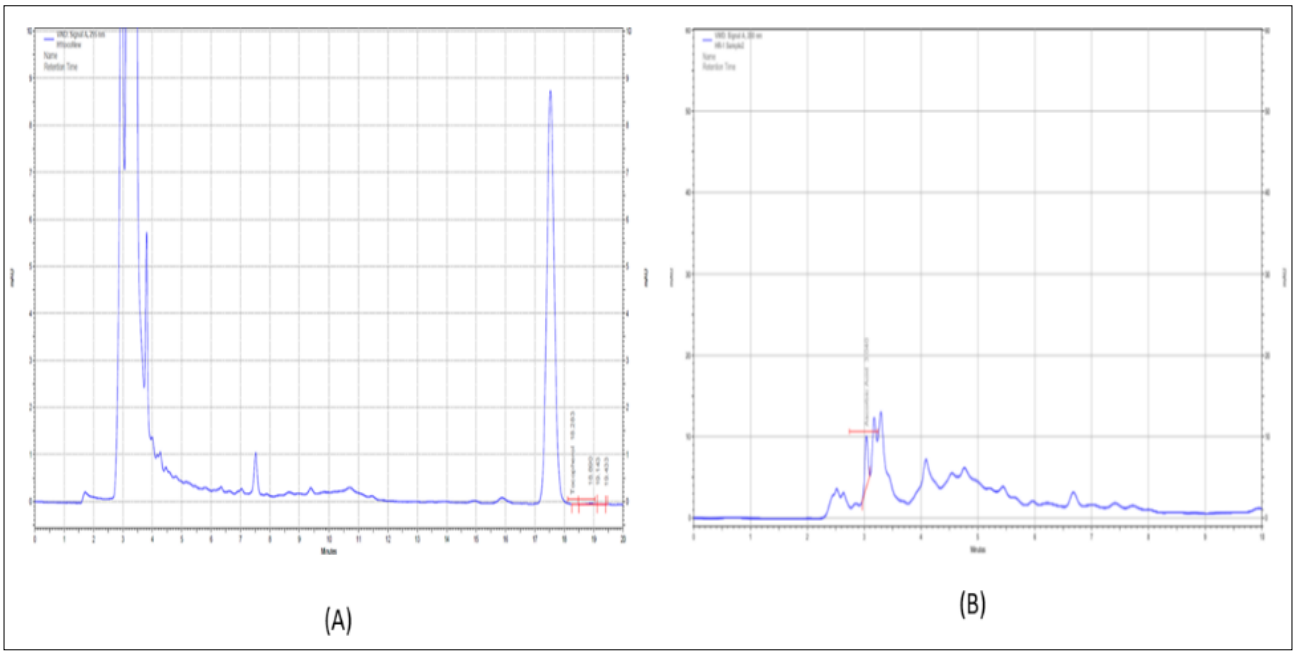

Fig. 10. Showing chromatograph of: A. Vitamin E; B. Vitamin C of methanolic peel extract of *Citrus limon*.

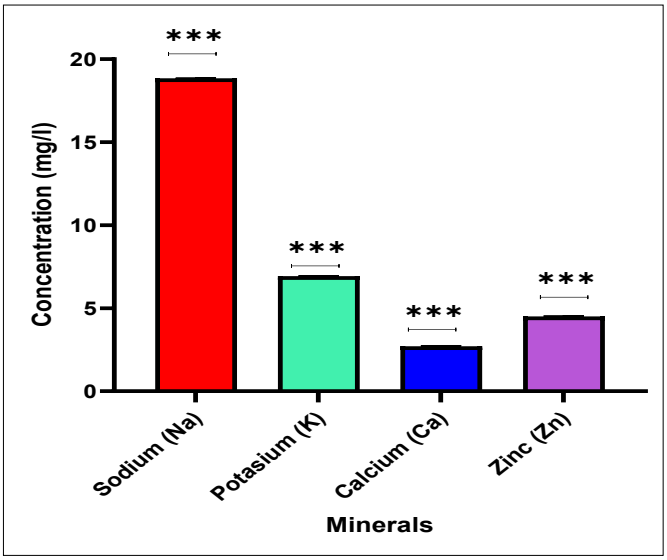

Fig. 11. Bar graph represents different concentration of minerals. All the values of the elements are statically significant using Duncan's multiple range test (DMRT), where $^{***}p<0.001$ (n = 3).

attribute that interacts with various biological targets to exert therapeutic effects, as these bioactive compounds are enriched with natural antioxidant potency (30). Within the scope of phytomedicinal therapies, citrus fruits are increasingly recognised for their high levels of phytochemicals, which contribute to their notable antioxidant efficacy. In the current investigation, we explored a multidisciplinary approach, including integrating phytochemical profiling, antioxidant screening through *in vitro* assay, MTT-based cytotoxicity and nutrient and mineral content analysis in HPLC mode from the methanolic peel extract of Kaji Nemu. Furthermore, we also demonstrate the GC-MS screened *in silico* modelling, which suggests the potential of Kaji Nemu peel-derived phytocompounds as modulators of thyroid protein (TRβ1).

The percentage yield of the methanolic peel extract was determined to signify the effectiveness of the extraction process and gives preliminary data on the composition and potential of the extract. The extract showed a relatively high extraction yield of 10.39 %. Similar findings have been reported in previous studies, where the methanolic peel extract of Kaji Nemu exhibited an extraction yield of 18.05 %, while another study reported a yield of 14.0 % for lemon peel extract (31). These observations suggest that methanol is an effective solvent for extracting phytochemicals from citrus peel. A higher extraction yield generally indicates the presence of a greater number of bioactive constituents in the plant material, highlighting its potential as a promising source for plant-based therapeutic or nutraceutical products (32).

Preliminary qualitative phytochemical analysis was conducted on the prime phytochemicals from the methanolic peel extract of Kaji Nemu. The present study demonstrated the existence of tannin, phenol, saponins, flavonoids, alkaloids, terpenoids, carbohydrates and glycosides, where phenol, alkaloid, saponin and flavonoid were highly present in the methanolic peel extract. This is consistent with an earlier study, which reported that the methanol extract of lemon peel exhibited high presence of alkaloids, flavonoids, polyphenols, terpenoids, glycosides, saponins and tannins (12). Similarly, extracts from lemon peel using different solvents showed a significant amount of phenol, tannin, saponin, flavonoid, alkaloid, etc. Given that, the polar compound was more exposed than the non-polar compound to the phytochemicals present in Kaji Nemu, where methanol solvent efficiently dissolved bioactive components and has heating stability, which could retain volatile compounds in the extract (12). Flavonoids, phenols, terpenoids and tannins were a prevalent group of phytochemicals identified in Kaji Nemu, which exhibited potent antioxidant characteristics. Moreover, terpenoids are the major group of bioactive compounds, including camphor, citral, carotenoids, etc., which contribute to citrus aroma, flavour and also show antioxidant and maintain thyroid follicular cells by mitigating ROS-induced damage (32). Tannins are also under the class of polyphenols known for their anti-inflammatory, antioxidant activities and modulating NADPH oxidase or free radicals and enhance thyroid hormone production (33). Additionally, alkaloids are also applicable for pharmacological activities, including antimalarial, respiratory or cardiac stimulants, antioxidative and also preventing thyroid cell proliferation in thyroid cancer (34).

Flavonoids, phenols and tannins are considered the most promising, prime and quantifiable phytochemical compounds. Quantitative phytochemical analysis of the methanolic peel extract

revealed substantial levels of total phenols, flavonoids and tannins. Among these compounds, tannins were present in the highest concentration, followed by phenols and flavonoids. This finding is consistent with previously reported studies on the quantitative estimation of TPC, TFC and TTC. Earlier studies have reported TPC and TFC values in different varieties of lemon peel as 20 ± 2.9 mg GAE/100 g, 5.47 ± 0.26 mg QE/100 g, 35.0 ± 0.67 mg GAE/g, 14 ± 2.3 mg QE/g, 27.14 ± 0.23 mg GAE/g and 15.96-32.7 mg QE/g, respectively (35).

Similarly, the TTC reported in citrus peel extracts varies widely in the literature. Reported values include 12.02 ± 0.7 mg/g, 2.02 ± 0.01 µg TAE/mg extract, 0.41 ± 0.01 µg/100 µL and 0.64 ± 0.03 mg/g, depending on the plant variety and extraction conditions (36). Previous studies have reported that locally grown lemon peel varieties contain higher amounts of phenolic and flavonoid compounds when extracted using methanol compared with other solvents. Variations in the reported values of TPC and TFC may be attributed to several factors, including plant species distribution, environmental conditions, developmental stage, harvesting time and extraction methods such as solvent type and extraction duration (37). These factors significantly influence the accumulation of bioactive compounds in plants. Phenolic and flavonoid compounds contain hydroxyl groups (-OH) that contribute to strong redox properties, enhancing their antioxidant activity (36, 37). These compounds can neutralise free radicals and chelate metal ions that catalyse the formation of ROS, thereby preventing lipid peroxidation. Polyphenols are also associated with various therapeutic activities, including antioxidant, antibacterial, antiviral, anti-inflammatory and anti-allergic effects. Additionally, phenolic compounds may influence thyroid hormone biosynthesis through modulation of TPO activity and activation of the Nrf2 signalling pathway, which helps reduce oxidative stress in thyroid cells. Flavonoids such as eriocitrin, hesperidin, caffeic acid and naringenin have also shown potential antithyroid activity by regulating TSH receptor signalling and thyroid hormone transport (37, 38). Tannins, another class of polyphenols, exhibit strong antioxidant, anti-inflammatory, antibacterial and antithyroid properties (39). More than one antioxidant assay was added together to obtain more consistent information about the bioactive compounds in the target extract and their associated antioxidant potential (40). Overall, the high phenolic and flavonoid content observed in the present study suggests that the extract may serve as a promising natural antioxidant source for nutraceutical and pharmaceutical applications.

The antioxidant capacity of the methanolic peel extract of Kaji Nemu was particularly evident in the DPPH assay, which demonstrated strong radical scavenging activity with an IC_{50} value below 50 µg/mL. According to established criteria, extracts with IC_{50} values between 50–100 µg/mL are considered to possess strong antioxidant activity, while extracts with IC_{50} values greater than 250 µg/mL are considered weak antioxidants (41). The strong antioxidant activity observed in the present study is comparable with previous studies on lemon peel extracts, which reported IC_{50} values ranging from 69.29–97.33 µg/mL in methanolic extracts (11, 42).

The FRAP assay further confirmed the strong antioxidant capacity of the extract by measuring its ability to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). This reduction occurs through the electron-donating capacity of antioxidant compounds present in the extract. In the present study, the absorbance of the peel extract increased significantly due to the formation of Fe^{2+}, indicating strong reducing power (43). Similar antioxidant activity has been reported in other citrus species, including *Citrus sinensis* and *Citrus reticulata*, where antioxidant activity correlated with high phenolic and flavonoid content (44). Therefore, these results indicate that Kaji Nemu peel extract contains potent natural antioxidants capable of neutralising free radicals and preventing oxidative damage.

The bioactive compounds in Kaji Nemu extract may either trigger an intracellular enzyme that accelerates thyroid hormone formation or substantially control it. The study declares a flavonoid compound termed kaempferol in Kaji Nemu elevates the efficiency of T_3 biosynthesis by 2.6-fold. Furthermore, *Citrus* sp. extracts endogenous ingredients might possess an anti-oxidant impact on the release of thyroid hormone. The phenolic substances of the Kaji Nemu plant contribute to its antioxidant properties. According to studies, flavonoids extracted from Kaji Nemu plants might affect the hypothalamus-pituitary feedback mechanism since these compounds are structurally analogous to T_3 and T_4 (19, 39).

Thyroid-stimulating hormone is a heterodimeric hormone with a glycoprotein structure like thyroxine (T_4) and triiodothyronine (T_3) that coordinates thyroid homeostasis via engaging with the TSHR. Thyroid-stimulating hormone receptors is mostly expressed in thyroid follicular cell where it regulates their growth and activity. Thyroid-stimulating hormone receptors agonist is currently used in patients with thyroid cancer, where, in certain conditions, the HEK293 cell line steadily expresses TSHR such as TRβ1. HEK293 cells act as a model system for assessing the impact of chemical compounds or pharmaceuticals on thyroid-associated transporters, particularly the sodium-iodide symporter (NIS/SLC5A5), which is critical for iodide transport (14). Some studies declare cytotoxicity effects of citrus extracts (*Citrus aurantium* (bitter orange), *C. maxima* (pomelo) and *C. medica* (citron) such as MCF-7, HL60 cells and MDA-MB-231 breast cancer cells, showing IC_{50} 83, 320 and 10 µg/mL (44). However, there is no strong evidence which reveals the anti-thyroidal cytotoxicity effect of Kaji Nemu peel extracts. The present study can be considered to be the first of its kind, which reveals the cytotoxicity effect of peel extract of Kaji Nemu on the HEK293 cells with an IC_{50} value observed at 177.7 µg/mL. According to the National Cancer Institute and Geran Protocol, chemicals/extracts are classified by IC_{50} as follows: ≤ 20 µg/mL, highly toxic; 20–200 µg/mL, moderately toxic; 201–500 µg/mL, weakly toxic; >500 µg/mL, non-toxic to cells (45). The estimated IC_{50} value for the extract falls within the range of 20–200, indicating moderate cytotoxic effects against the cells. Therefore, this indicated that peel of Kaji Nemu could be a promising candidate for their protective effects against the cell line.

GC-MS analysis was performed due to the high content of TPC and TFC and significant antioxidant activity was observed in the studied extract, indicating a wide range of bioactive components that merit further exploration of chemical analysis. Butanamine, 2,2-dinitro-N-methyl, a novel compound isolated from endophytic bacteria present in the Kaji Nemu, contributes a promising antibacterial property in terms of inhibiting pathogens and can be used as a possible drug in the future. In the present study, GC-MS screening demonstrated that Kaji Nemu peel extract is a rich source of many significant naturally synthesised volatile chemical compounds. GC-MS screening presents 3 novel chemical compounds with high peak area percentage along with their significant pharmacological activities that were first reported in MPECL. This offers a pioneering investigation for this work, including 11-Oxatetracyclo [5.3.2.0(2,7).0(2,8)] dodecan-9-one, 1H-Tetrazole,

5-(3,4-dimethoxyphenyl) and 2-Methyl-Z, Z-3,13-octadecadieno, respectively. The rest of the chemical compounds identified with their high peak area percent could align with previously reported studies, underscoring the potent pharmacological activities (7). 11-Oxatetracyclo [5.3.2.0(2,7).0(2,8)] dodecan-9-one falls under the sesquiterpenoid derivatives lactone known for its antinociceptive and anti-inflammatory properties, while 6-Methoxychroman-2-one, a derivative of coumarin, has been reported in a previous study for other lemon varieties, expanding the biological applications, including antioxidant, stimulating apoptosis in U-937 leukaemia cells and anti-inflammatory (46). Moreover, this compound offers a modulatory effect on hyperthyroidism by preventing excess TPO and thyroid hormone production. Furthermore, the presence of epoxides and their derivatives also highlights the utility as anticancer and antiviral drug formulations. Interestingly, 1H-Tetrazole, 5-(3,4-dimethoxyphenyl), a tetrazole derivatives presence revealing the promising antioxidant and antitumor effects linked to oxidative stress. As there are no detailed reports on the beneficial effects of these compounds in thyroid-related disorders, a few studies have documented that tetrazole, coumarin and terpenoid scaffolds exert modulatory effects by regulating thyroid-associated gene expression (47). Pentadecanoic acid, N- hexadecenoic acid and octadecanoic acid are a class of palmitic fatty acids, identified as potent antioxidants and lipid-lowering properties, which are beneficial for thyroid conditions such as autoimmune thyroiditis, hypothyroidism, etc. (43). Hence, outcomes of this work underscore the ethnomedicinal value of peel extract of Kaji Nemu as a multitarget therapy.

The virtual screening and molecular docking analyses offered additional insights into the therapeutic potential of GC-MS screened phytocompounds. The identified lead candidates demonstrate superior binding affinity with the selected thyroid-related target, that is TRβ1, exceeding the reference drug methimazole in binding energy (-4.0 kcal/mol). PubChem ID 8456 (3-tert-Butyl-4-hydroxyanisole) recorded -6.7 kcal/mol with TRβ1, demonstrating promising predicted interactions to its respective targets. This finding indicated that Kaji Nemu-derived bioactive compounds could modulate receptor-mediated signalling and a broader therapeutic spectrum compared with methimazole. The vital function of THRβ1 in controlling the hypothalamus-pituitary-thyroid system as well as biochemical processes, especially metabolism, liver and kidney functions, etc., reveals that receptor abnormalities are the fundamental root cause of an array of disorders. 3-tert-Butyl-4-hydroxyanisole is a phenolic compound having potential antioxidant-rich properties associated with endocrine modulation, anticancer, antibacterial and anti-inflammatory effects (48). In accordance with our previous study, the stronger binding energy of the lead compound with thyroid-linked proteins (TPO, TRα1 and TRβ1) in comparison to the reference compound methimazole revealed in the present study supports the possibility of the compound to provide a therapeutic role in anti-thyroidism, which, however, needs further in-depth understanding (26).

Following GC-MS profiling, HPLC analysis showed that the peel extract is a rich source of antioxidative nutrients, particularly vitamin E and C. The detection of these vitamins in the extract is consistent with previous studies on other lemon species, which reported concentrations of approximately 0.15 mg/mL for vitamin E and 53 mg/mL for vitamin C, respectively. Both vitamin E and C are potent natural antioxidants with important clinical benefits. These vitamins often act synergistically in the lipid and aqueous phases, respectively, protecting against oxidative damage, including lipid peroxidation in cells and tissues. In addition, they may help regulate inflammatory responses by modulating signalling pathways such as NF-κB (47). Vitamin C from Kaji Nemu has also been reported to support skin immune defence by protecting against UV-induced damage and participating in anti-apoptotic mechanisms. Furthermore, *in vivo* studies suggest that vitamins E and C may act as pro-oxidants in certain cancer therapies and may also contribute to the regulation of thyroid hormone biosynthesis in thyroid dysfunction (49). Therefore, these findings indicate that the peel of Kaji Nemu, often considered a waste product, is a valuable source of vitamins with promising nutritional and therapeutic potential.

Mineral analysis further demonstrated the presence of essential elements such as sodium, potassium, calcium and zinc in the extract. Sodium and potassium are involved in maintaining electrolyte balance and may facilitate iodine transport into thyroid cells through activation of the sodium-iodide symporter (NIS). Calcium plays an essential role in bone formation and cellular signalling, while zinc acts as an important antioxidant and anti-inflammatory element by activating Nrf2-mediated cellular responses and regulating thyroid hormone metabolism (50, 51). Recent scientific investigations on Kaji Nemu have primarily focused on plant health, antimicrobial potential and horticultural characteristics rather than its endocrine-related pharmacological properties. For instance, a recent study investigated the occurrence of *Citrus tristeza* virus infection in Assam lemon trees of varying ages (3–25 years) using molecular diagnostic tools, highlighting the importance of disease monitoring for citrus cultivation (52). In addition, phytochemical investigations of Kaji Nemu peel essential oil revealed the presence of bioactive volatile compounds exhibiting antimicrobial activity against pathogenic bacteria, including *Escherichia coli* and *Enterococcus faecalis* (53). Similarly, research reported that the isolation of plant-derived compounds from Assam lemon with significant inhibitory effects against microbial pathogens, indicating its potential role in natural antimicrobial formulations (54). Beyond pharmacological studies, horticultural research has also examined morphological traits, seed characteristics and biochemical parameters of Assam lemon to support breeding strategies and conservation of this indigenous cultivar (55). Furthermore, a recent review summarised the nutritional composition and phytochemical profile of Assam lemon, emphasising its potential functional and nutraceutical value (56). Despite these contributions, systematic investigations focusing on thyroid-related pharmacological potential of Kaji Nemu peel remain limited.

A key novelty of the present study lies in the comprehensive and integrated investigation of the methanolic peel extract of Kaji Nemu, focusing specifically on its potential modulatory effects on thyroid-related molecular targets. Although citrus species have been widely studied for their antioxidant and pharmacological properties, systematic investigations exploring the therapeutic relevance of Kaji Nemu peel in the context of thyroid dysfunction remain extremely limited. To the best of our knowledge, this study represents the first integrated approach combining phytochemical profiling, antioxidant evaluation, cytotoxicity assessment, nutritional analysis, GC-MS-based compound identification and structure-based *in silico* screening against TRβ1 using the peel extract of Kaji Nemu. This multidisciplinary framework provides a more comprehensive understanding of the potential therapeutic relevance of this underexplored citrus variety.

The strong antioxidant activity observed in the DPPH, FRAP and ABTS assays may be largely attributed to the high levels of phenolic compounds, flavonoids and tannins identified in the extract. These phytochemicals are well known for their ability to donate hydrogen atoms or electrons, thereby neutralising reactive oxygen species and reducing oxidative stress. Since oxidative imbalance plays a critical role in thyroid dysfunction-particularly in hyperthyroidism, where excessive reactive oxygen species are generated-natural antioxidants derived from plant sources may contribute to restoring cellular redox balance and protecting thyroid follicular cells from oxidative damage. Interestingly, the cytotoxicity assay revealed moderate cytotoxic activity of the extract against HEK293 cells with an IC_{50} value of 177.7 µg/mL. This observation can be interpreted in the context of the phytochemical composition of the extract. Many phenolic and terpenoid compounds present in citrus peels exhibit dual biological roles, acting as antioxidants at lower concentrations while exerting cytotoxic or growth-inhibitory effects at higher concentrations. Therefore, the moderate cytotoxic activity observed in this study may reflect the presence of bioactive compounds capable of modulating cellular metabolic pathways. Importantly, the IC_{50} value obtained falls within the range classified as moderately cytotoxic according to National Cancer Institute criteria, suggesting that the extract retains biological activity without exhibiting excessive toxicity.

Furthermore, GC-MS analysis revealed a diverse array of phytochemicals, including terpenoids, sesquiterpenoids, coumarin derivatives and fatty acids, several of which have been previously associated with antioxidant, anti-inflammatory and endocrine-modulating activities. Among the identified compounds, 3-tert-butyl -4-hydroxyanisole emerged as a promising candidate during the molecular docking analysis, demonstrating a stronger predicted binding affinity toward TRβ1 than the reference antithyroid drug methimazole. The observed hydrogen bonding and hydrophobic interactions within the receptor binding pocket suggest that this compound may contribute to the modulation of thyroid hormone receptor signalling.

Conclusion

This study provides a comprehensive preliminary evaluation of the bioactive potential of the methanolic peel extract of Kaji Nemu (*Citrus limon* (L.) Osbeck), highlighting it as a promising source of phytochemicals, antioxidant compounds and nutritionally relevant constituents. Strong antioxidant, anti-inflammatory, antibacterial and antithyroid properties were attributed to the abundance of potent bioactive compounds, as evidenced by qualitative and quantitative analyses. *In vitro* assays demonstrated significant free radical scavenging capacity and moderate cytotoxic effects against HEK293 cells, indicating potential therapeutic relevance while providing preliminary insights into its biological safety profile. Furthermore, this study integrates phytochemical profiling, biological evaluation and structure-based *in silico* analysis of an underexplored yet culturally significant citrus variety. Such an approach not only contributes to the scientific understanding of Kaji Nemu but also highlights the pharmaceutical and nutraceutical potential of indigenous plant resources from Assam. Although the present findings demonstrate promising *in vitro* and computational outcomes, further studies involving compound isolation, *in vivo* validation and mechanistic investigations are necessary to fully elucidate its therapeutic potential and support future pharmaceutical development.

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Authors' contributions

RY conceived the study, performed the experiments, conducted data analysis and prepared the manuscript. RA assisted in performing the experiments and provided the technical mentorship for performing the experiments and data analysis. JCK supervised the study and contributed to manuscript preparation. SYJ revised the manuscript and edited the figures. All the authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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