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Structural Prediction of Bioactive Compound Elucidated Through ^{1}H and ^{13}C NMR Based Metabolomics from *Physalis Minima*

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ABSTRACT

This study was performed to extract, purify, and characterize the bioactive compound from the ethanol extract of Physalis minima fruit. Furthermore, the free radicals scavenging potential of that purified and characterized test compound was investigated through in-vitro antioxidant studies. The study's findings demonstrated that the ethanol extract of Physalis minima fruits is supposed to contain many critical phytochemical constituents. The predominant fraction acquired from the extract via column chromatography and TLC was characterized using ^{13}C NMR and ^{1}H NMR techniques. The analyses results revealed that the chemical structure and name of the bioactive compound are 2-(3,4-dihydroxyphenyl) - 3, 4,4a 5-dihydro-2H-chromene-3,5,7-triol.

Keywords

Physalis minima, Bioactive compound, ^{1}H NMR, ^{13}C NMR.

Introduction

Herbs and medicinal plants are widely regarded as important sources of medicinal properties worldwide. The present situation in the pharmaceutical companies demonstrates that medicinal plants are managed to grow for more development ventures by the pharmaceutical industry [1]. The India is blessed with numerous plant biodiversity, certain numbers of them possess fine bioactive molecule with a variety of biological activities such as free radical scavenging, antimicrobial, anti-inflammatory, as well as anticancer properties [2]. Such distinguishing characteristics have boosted their potential as precursor compounds for drug discovery and development. Furthermore, Indian herbs have played a crucial social-economic role in developing nations [3]. For several generations, traditional medicine has been widely used by the local populations to treat a wide range of human health discomforts. Furthermore, natural (organic) ingredients are much less harmful than synthetics, which makes them quite preferable and promising [4]. Plant-derived phytochemicals are non-nutritional substances that are required for proper human biological processes. Such compounds have been found in plants, and their products can act as natural antioxidants. Such antioxidant compounds are reliable and can safeguard macro-molecules (proteins, nucleic acids, lipids, and so on) in cells from oxidative damage by free radicals in multiple biochemical processes, which take place in the body [5]. The interaction of ROS with macromolecules can causes apoptosis, which can lead to a variety of biological, neurobiological, and myocardial infarctions. An oxidation stability among human antioxidant defensive measure as well as ROS generated during cellular biochemical processes is required for proper biological processes [6]. For instance, continuous stress consumes more energy from elevated metabolism mechanisms, favouring ROS production in the muscle [7]. Thus, a change in equilibration among subcellular antioxidants and ROS occurs, resulting in oxidative stress and possibly oxidative stress - induced to cells, organs, and tissues [8]. These lead to cause severe chronic health issues. Antioxidants protect tissues and organs from free radical damage by inhibiting the synthesis and accumulation of radicals, scavenging those, or helping to promote their degradation. Synthetic antioxidants have generally been discovered to be harmful to human health [9]. Hence, there has been an increase in the search for effective, non-toxicity bioactive substances with antioxidant properties in recent times. Several phytoconstituents, including tannins, flavonoids, phenolic acids, steroids, ascorbic

acid, saponins, glycosides, and tocopherols, have been shown to possess antioxidant activity, suggesting the possibility of their use in the cure of numbers of chronic conditions such as diabetes, neurodegenerative, atherosclerosis, hepatitis, cancer , and others [10]. *Physalis minima (Miniature Physalis)* Linn, a member of the Solanaceae family, seems to be widely available around the tropical and subtropical regions, where it grows naturally as bush and perennial herbs [11]. *Physalis minima* Linn has been used in Indian and Chinese folk medicinal treatments to reduce fever, colds, coughs, and asthma, among other things, because it contains anti-microbial and anti-inflammatory properties [12]. Since this plant has been reported as possessing pharmaceutically valuable phytochemicals. For example, steroids, tannins, phenolics, and alkaloids serve as anti-neoplastic and antimicrobial agents [13]. Furthermore, the bioactive compounds present in this plant have been reported earlier as possess remarkable analgesic, anti-cancerous, anti-inflammatory, anti-diabetic, anti-pyretic potentials [14].The oxidants are the most responsible for aging and other life-threatening health issues such as cancer, compromised immune system, etc. The maintenance of free radicals in human physic is an essential factor to attain an extended lifespan [15] Such bioactive molecules derived from medicinal plants are receiving great research interest, however their extraction and characterization of phytochemicals from plants are unique challenges [16]. Researchers have developed numerous procedures for extracting bioactive components to improve the accomplishment and efficiency of crude drugs used to treat illness [17]. Much attention is being paid to optimizing the mode of extraction of bioactive phytoconstituents to reduce the cost associated with fabrication and separation. Thus, the extraction of bioactive components from plants necessitates the use of suitable extraction strategies and processes that yield extracts and fractions rich in bioactive components. Thus, the extraction methods have a significant impact on yield, the nature of phytochemical content, and so on. Hence, this research was designed to extract and structural identification of bioactive compounds from *Physalis minima* using standard separation and characterization techniques. Furthermore, their free radicals scavenging potential was analysed through typical *in-vitro* approach.

Materials and Methods

Collection, Identification, and Authentication of sample

The fruit of *Physalis minima* was collected from Kolli Hills in Namakkal District of Tamil Nadu from October to December 2020.

Preparation of plant extract

About 2kg of *Physalis minima* fresh fruits were collected and washed with sterile distilled water, and then they were sliced into pieces and dried shadow for 12 days at room temperature. Subsequently, the well-dried fruit sample was pulverized with an electric pulveriser and the powdered sample was refrigerated for further processing. About 100g of well-powdered fruit sample was dissolved in 250 ml of ethanol solvent in a 500 ml conical flask and kept in an orbital shaker for 48 h for the extraction process. After extraction, the extracts were individually filtered through Whatman No.1 filter paper, excess solvent was desiccated, and obtained extract was concentrated and used for further studies.

Phytochemical analysis

The qualitative analyses of essential phytochemicals such as Flavonoids, glycosides, terpenoids, steroids, anthraquinones, tannins, proteins, alkaloids, and carbohydrates contents of ethanol solvent extract were investigated with specific standard protocols.

Separation of major bioactive compound from the extract

A standard column chromatography procedure was followed to separate the major bioactive compound from the ethanol extract. In brief, the crude ethanol extract was loaded on a silica gel-filled cylindrical glass column. The column was packed with hexane and eluted by increasing the solvent polarity from hexane to ethyl acetate at various ratios (Hexane: ethyl acetate: 100, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, and 100). Subsequently, the typical thin-layer chromatography (TLC) was performed to elute a compound from the column chromatography fractionated substances by placing in the silica gel (60–120 mesh) loaded TLC plates of 20 x 20 cm. The fractions were then taken in the TLC plates of ½ inch through the capillary tube and placed at the chamber until the solvent reached the top portion of the plate. The plates were dried up and the solvent front was marked. The chromatophores were noted and with specific spray reagent and the standard formula was used to follow the Rf value through visual detection.

^{1}H and ^{13}C -NMR analyses

From the TLC plate, single fractions (compound 1 derived from 90:10 ratio respectively in column chromatography) were taken and subjected to structural determination and identification based on the type of proton or hydrogen by the analytical method called ^{1}H and ^{13}C -NMR spectroscopy analyses. In NMR, the standard sample loading and operative protocol was followed, with the intensity of peaks being directly proportional to the number of nuclei. The ^{1}H-NMR and ^{13}C-NMR spectra provide information about the number of protons in a compound and the properties of proton form.

Free radicals scavenging potential analysis

The free radicals scavenging potential of separated compound 1 was investigated with various forms of free radicals (DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate), Nitric oxide, FRAP, H_2O_2, and reducing assays) through the *in-vitro* approach. The different dosages (20 - 80 µg/ml) of ascorbic acid (vitamin C) was considered as a reference.

FRAP Assay

Total antioxidant activity of the test compound was studied through FRAP assay by following the standard protocol. About 2 ml of the dosage above test compound was added with 2 ml (individually) of phosphate buffer (0.2 M) as well as potassium ferricyanide (1%). These reaction mixtures in triplicates were incubated for 25 min at 45°C. Subsequently added 2 ml of trichloroacetic acid (10%) and spun for 10 min. Then added2.5 ml (each) of deionised water and supernatant, 0.5 ml of ferric chloride (0.1%) solution. The absorbance of the test and positive control was read at 700 nm.

DPPH· Assay

The DPPH free radicals scavenging potential of compound 1 with various dosages (20- 80 µg/ml) was analyzed with standard assay protocol. Approximately 100 µL of various dosages of test compound were mixed with 100 µL of DPPH reagent and kept undisturbed for 30 min at room temperature (RT). Subsequently, test and positive control absorbance was read at 517 nm. The percentage of DPPH scavenging and corresponding IC_{50} values were calculated using the typical formula and linear regression analysis.

Nitric oxide (NO) scavenging assay

NO scavenging efficiency of bioactive test compound was studied through following a typical methodology. Approximately 0.5 ml of phosphate buffer (pH 7.4) and 2 ml of 10 mM sodium nitroprusside were added with 1.5 ml of the concentrations mentioned aboveto test the bioactive compound. Such reaction mixtures were incubated at RT for 100 min. After incubation, about 1ml of sulfanilic acid was added and kept for 5 min. Then, 1 ml of naphthylethylenediaminedihydrochloride was added and kept undisturbed for 30 min. The absorbance of such reaction mixtures was read at 540 nm. The percentage of scavenging and IC_{50} values were determined by following typical formula and regular linear regression analysis.

Reducing power assay

The regular and specific protocol was followed to evaluate the ferricyanide reducing the potential of bioactive test compound with the abovementioned concentrations. About 1 ml (each dosages of sample) added with 2.5 ml (individually) of phosphate buffer and $C_6N_6FeK_3$ and kept in a water bath (50°C ± 2) for 25 min. Then 2.5 ml of TCA was blended and then spun at 3500 g for 15 min. Subsequently, 2.5 ml of supernatant was mixed with 2.5ml of distilled water, and then added 0.1% of $FeCl_3$ (0.5 ml) solution. Then the absorbance of reaction mix was read at 700 nm.

Hydrogen peroxide assay

The standard hydrogen peroxide assay protocol was performed to evaluate the H_2O_2 scavenging potential of the test compound with the abovementioned concentrations. About 1.2 ml of the abovementioned concentration of test compound was blended with 0.8 mL of H_2O_2 (40 mM). Then, the reaction mixtures in triplicates were incubated for 10 min at RT. Then, the test and control absorbance was read at 230 nm through a UV-visible spectrophotometer. The percentage of scavenging and IC_{50} values were determined by following typical formula and regular linear regression analysis.

Results and discussion

Qualitative phytochemical analysis

The results obtained from the qualitative phytochemical analysis on an ethanol extract of *Physalis minima* fruits demonstrated the occurrence of essential phytochemicals including glycosides, terpenoids, anthraquinones, tannins, saponins, and alkaloids. Many researchers have reported such phytochemicals as they possess remarkable medicinal properties and serve as primary ingredients for more numbers of drugs. For instance, the plant-based terpenoids (p-menthanemonoterpenoids, ingenolmebutate, paclitaxel, cannabinoids, and so on) are bioactive compounds used for the treatment of antimicrobial, anticancer, analgesic, antihyperglycemic, antiparasitic, and anti-inflammatory. The glycosides derived from various plant species have been reported as possess remarkable antioxidant, hepatoprotective, anti-diabetes, antitumor, anti-inflammatory, and antimicrobial activities. Several literatures showed that the saponins group bioactive compounds possess significant anti-inflammatory, antimicrobial, hemolytic, anticancer, insecticidal, and antioxidant activities. Similarly, the plant based anthraquinones had been reported as antimicrobial, anti-inflammatory, and laxatives agents. The alkaloids (strychnine, nicotine, quinine, and morphine) were being used as excellent anti-inflammatory, cardioprotective, and anaesthetics agents.

Column and TLC chromatogram analyses

From the column, chromatogram number of fractions (15) were obtained and concentrated using a rotary vacuum evaporator. Similarly, Gini and JeyaJothi used column chromatography to separate the bioactive compounds of phenolic group from ethyl acetate extract of *Salviniamolesta*mitchell [18]. The fraction with increased quantity was subjected to TLC analysis. The number of intensity spots (15) was obtained from the TLC plate while exposed to the vanillin reagent (Figure 1). The fractions with the same number of spots with similar Rf values (considered compound 1) were found with hexane and ethyl acetate solvent proportion as 90:10 in TLC plate. Similarly, Sasidharan et al., reported that as used methanol and dichloromethane at 6:4 ratio to separate the major compound-containing bands [19]. In general, the bands with single clearcolor in TLC plate can be considered pure compound, and else if it contains a mixture of some other colors, it shows the presence of other components [20].

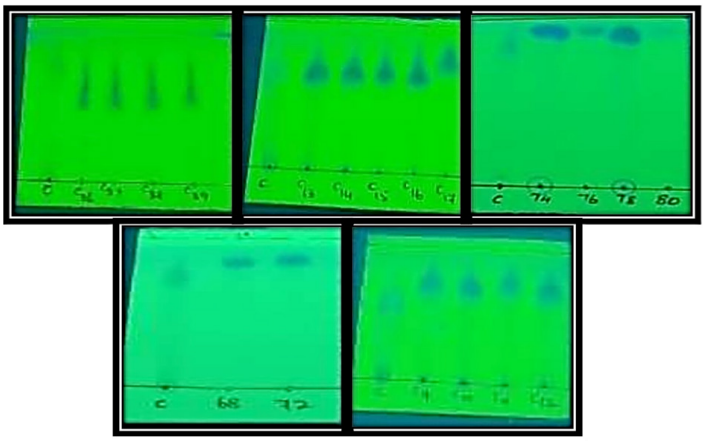

Figure 1: Thin layer chromatography analysis of column chromatography separated fraction.

Characterization of purified bioactive compound 1

The compound extracted from *P. minima* fruits and purified by subsequent column chromatogram and TLC was named as PHYMIN-22. Their chemical name and molecular formula were identified as 2-(3,4-dihydroxyphenyl)-3,4,4a,5-dihydro-2H-

chromene-3,5,7-triol and $C_{15}H_{16}0_6$ respectively through ^{13}C NMR and ^{1}H NMR techniques. Very few sharp peaks were found at 36 ppm, 44-71 ppm, 68.13-88.25 ppm, 102.23-120.45 ppm, and 171.83-175.35 ppm corresponding to CH in oxygen bearing ring, C-OH in chromene ring, C-OH in the ring system, Unsaturation in the ring, and =C-OH in aromatic ring respectively (Figure 2). Most of the peaks were related to the spectrum of toluene and isopropanol. Furthermore, the ^{1}H NMR spectrum analysis identified the presence of following groups. Number of spectrum were found at 1.32-1.57 ppm related to methylene, 2.0 ppm related to hydroxyl group attached to propyl ring, 2.7 ppm related to interlinked CH, 3.39 ppm corresponding to CH-OH interlinked aliphatic ring, 5.21 ppm states that attachment of OH to terminal dihydroxy phenyl ring, and 5.8, 6.0, 6.8, 7.0, 7.2 ppm were related to aromatic protons (Figure 3). Furthermore, the peaks found at 20.1 ppm, and 36 ppm related to terminal phenyl CH in the oxygen ring, similarly, the peaks found at 44, 48, 59, 64, 71 ppm states that carbon interlinked to OH ring, peaks at 68.13, 88.25 ppm revealed carbon to hydroxyl ring, and 102.33, 120.45 to alkene ring. This structural information clearly stated that the isolated PHYMIN-22 compound was confirmed as aforementioned compound.

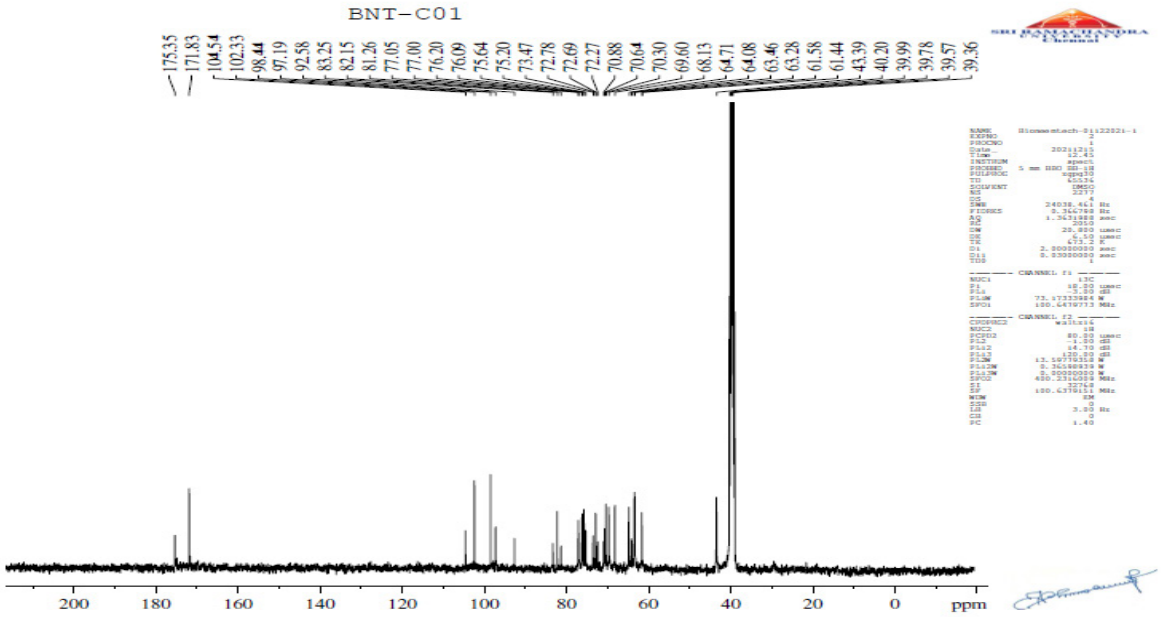

Figure 2: ^{13}C NMR Spectrum of purified bioactive compound: PHYMIN-22.

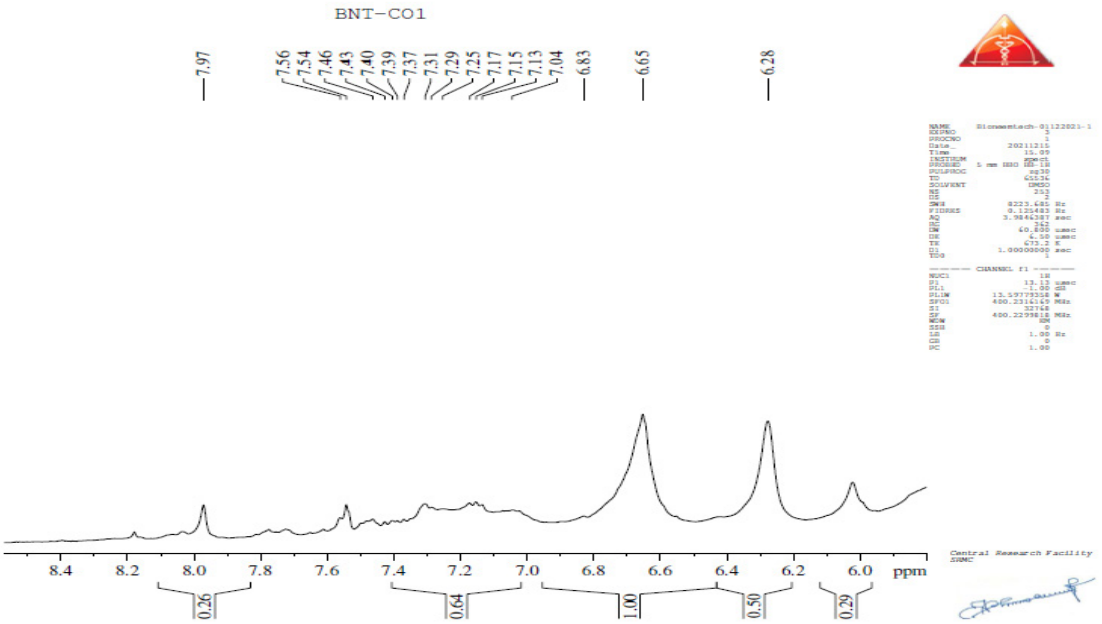

Figure 3: ^{1}H NMR Spectrum of purified bioactive compound: PHYMIN-22.

Free radicals scavenging potential

Total antioxidant and DPPH assay

The total antioxidant activity competence of identified bioactive compound was studied through FRAP assay. At a dosage of 80 µg/ml of the bioactive compound, the total antioxidant activity was 60% (Figure 4a) and the IC_{50} value was found as 26.28 µg/ml. These results were moderately comparable with the total antioxidant activity of ascorbic acid. This result strongly states that the purified bioactive compound have considerable total antioxidant activity. The obtained result also showed that the total antioxidant activity is directly related to the concentration of test compound. Similarly, the Yahia et al. reported that around 28 bioactive components belonging to the rutin and guinic acid of *Z. mauritiana leaf extract possess significant* total antioxidant activity [21]. The total phenolics, anthocyanins, tannins, and flavonoid groups based on bioactive compounds can effectively scavenge various forms of free radicals [22]. Similarly, the hydro-methanol *P. granatum*leaf extract showed considerable total antioxidant activity due to the presence of significant quantity of phenols [23].

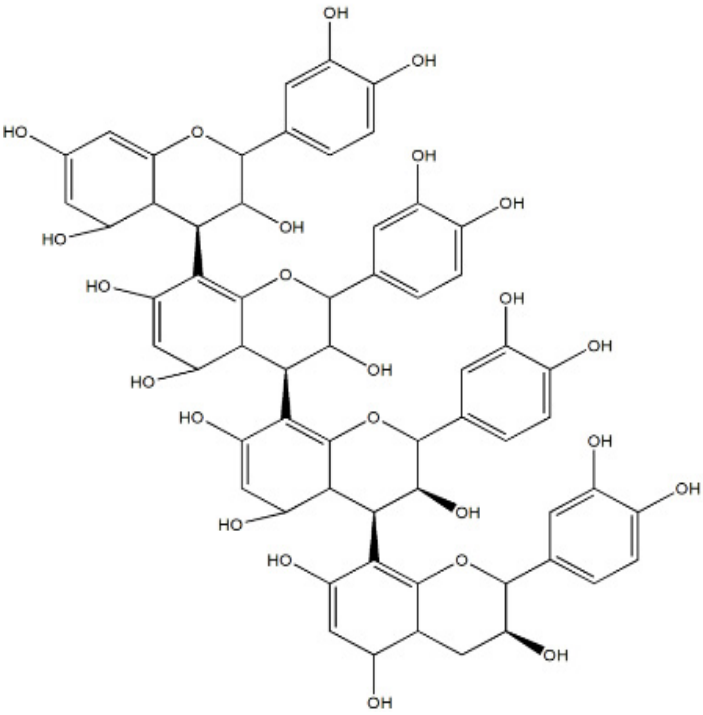

Figure 4: Structural prediction of bioactive compound 1 – PHYMIN-22.

DPPH radical scavenging assay

The bioactive compound extracted from ethanol extract of *Physalis minima* demonstrated dose-dependent DPPH radicals scavenging activity. Since, at increased concentration of 78 µg/ml, the bioactive test compound showed 81% of DPPH scavenging activity (Figure 4b) and IC_{50} value was found as28.35µg/mlInterestingly, the obtained DPPH scavenging result was close the DPPH scavenging potential of positive control ascorbic acid (82%). The test bioactive compound can donate an electron atom to DPPH radical and convert it to a violet coloured product as 1, 1-diphenyl-1, 2-picryl hydrazine. The α-carotene and lutein derived from fresh orange maize hybrids demonstrated considerable DPPH scavenging activity [24]. Similarly, the Kadum et al., separated and characterized the flavonoid and phenolic based bioactive compounds derived from *Phoenix dactylifera*showed considerable DPPH radicals scavenging potential in dose dependent manner. [25].

Nitric oxide radical scavenging assay

The purified test bioactive compound considerably scavenged nitric oxide radical up to 79% at 80 µg/ml concentration (Figure 4c), and IC_{50} value was calculated as 31.40 µg/ml. The bioactive test compound's obtained nitric oxide scavenging potential was considerably similar to the nitric oxide scavenging potential and IC_{50} value of ascorbic acid 84% and 35.88 µg/ml, respectively. Such nitric oxide scavenging potential was obtained in dose dependent manner. Generally, the nitric oxide radical is an active intermediate in many biochemical processes, for example, muscles relaxation, neurons signalling, inhibition of platelet aggregation, and regulation of cell mediated toxicity [26]. Phytochemical investigation uncovers that the seeds are a decent wellspring of flavonoids, phenolics, saponins, alkaloids and other secondary metabolites. The antioxidant capability of the green synthesized Liv-Pro-08 ayurvedic formulation was investigated by DPPH; FRAP measure, ABTS test, H 2 O 2 and NO scavenging assay. The biosynthesized NPs indicated fundamentally higher antioxidant activity [27]. This is a free radical that plays a role in a wide range of physiological functions such as neurotransmission, dilation of blood vessels, antimicrobial, and anticancer activity [28]. Habu and Ibeh reported the bioactive metabolite (ascorbic acid, cardiac glycosides, ketones, and so on) extracted and purified from leaf extract of *Newbouldialaevis* showed dose dependent nitric oxide scavenging activities [29]. Joseph and Ravi reported that the *Physalis minima* fruit extract possesses a considerable nitric oxide radicals scavenging activity and inhibits the acetylcholinesterase activity [30].

Reducing Power Assay

The purified test bioactive compound considerably reduced Fe^{3+} to Fe^{2+} up to 75% at 80 µg/ml concentration (Figure 4d) and IC_{50} value was found as 27.93 µg/ml. These values were almost close to the Fe^{3+} reducing potential of ascorbic acid. Here, the bioactive compound actively converts/reduce the colorless Fe^{3+} into intense blue color Fe^{2+}. Preferably, the phenolic compounds are actively involved in the reduction process. The methanol leaf extract of *Physalis minima* had been reported as possessing fine reducing power at 800 µg/ml concentration and it was dose dependent [31]. At increased concentration, the percentage of reducing potential also considerably increased. Another study reported that the *Physalis minima* leaf extract had remarkable reducing power potential [32]. The flavonoids and phenolic-based components can effectively involve in the radicals reduction process [33].

H_2O_2 Scavenging potential

The dose dependent H_2O_2 radicals scavenging potential for the test bioactive compound. At increased concentration (80 µg/ml), the

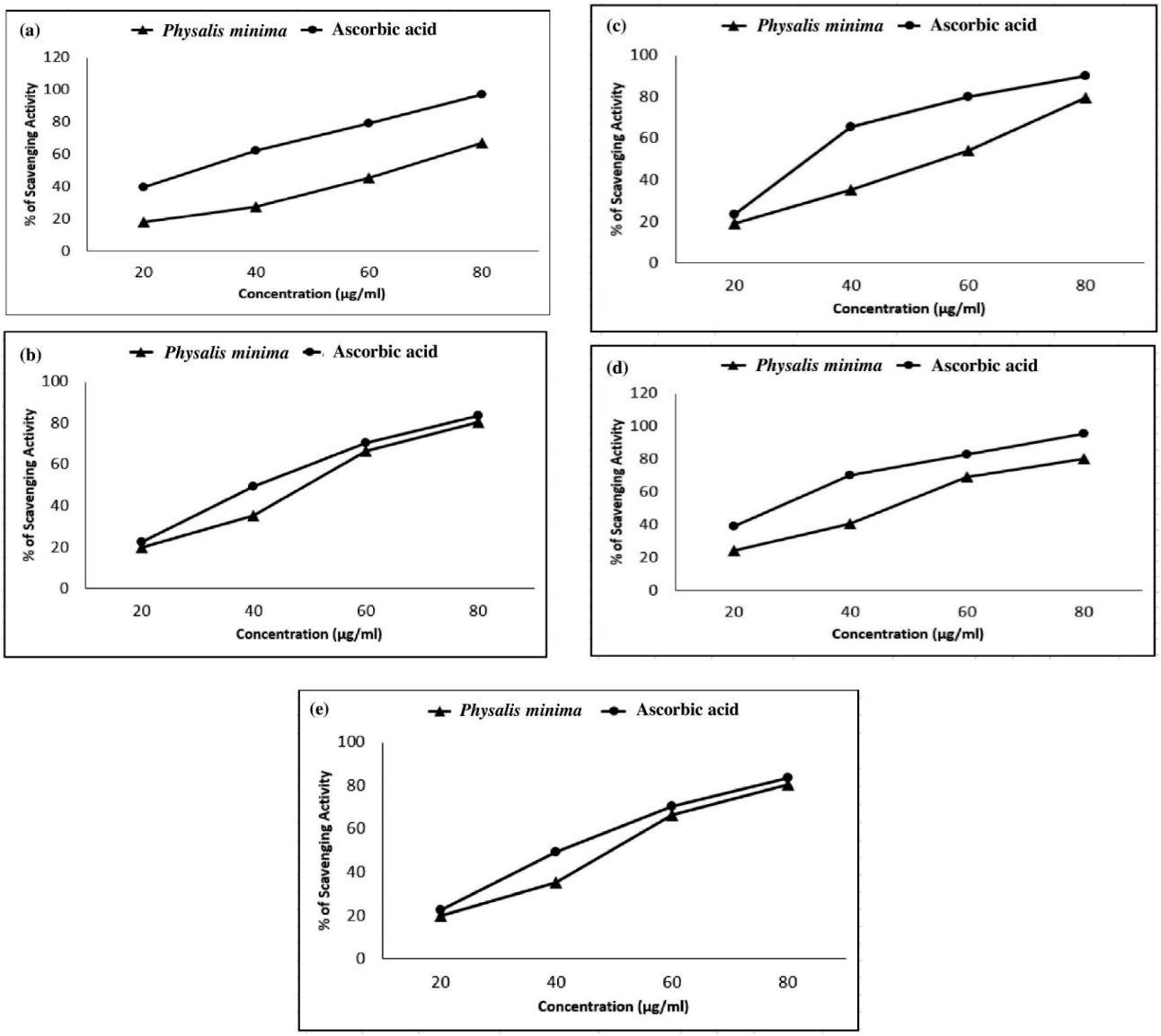

Figure 5: Free radicals scavenging potential of PHYMIN-22 compound: *In-vitro* analysis.

(a): Total antioxidant assay through FRAP **(b):** DPPH Scavenging assay **(c):** Nitric oxide scavenging assay **(d):** Reducing assay **(e):** H_2O_2 scavenging assay. The mentioned values are mean and standard error of triplicates.

test bioactive compound scavenge the H_2O_2 radicals up to 80% and it was almost similar to the H_2O_2 scavenging potential of positive control (82%) (Figure 4e). The IC_{50} value of bioactive test compound was found as 30.25 µg/ml. Ramakrishna Pillai et al. reported that the dose dependent H_2O_2 scavenging potential ofethanol extract of *Physalis angulate* fruit and leaves. Furthermore, they reported that the fruit extract contained more flavonoid and phenol compounds than leaves. Hence, the bioactive compounds belonging to these phytochemicals effectively scavenge the H_2O_2 [34]. Another species, Physalisperuviana, also reported possessing considerable H_2O_2 scavenging activity. In general, the plant which possesses an elevated quantity of essential phytochemicals and bioactive compounds derived from such phytochemicals can considerably act as free radical scavenging agents [35]. The bioactive compounds, which possess considerable free radical scavenging potential can be considered as suitable agent or precursor molecule to prepare antioxidant drugs [36].

Conclusion

The results obtained from the entire study revealed that the ethanol extract of *Physalis minima* fruit contained a considerable number of essential phytochemical components. The major fraction obtained from this ethanol extract through the column chromatography and TLC was characterized through ^{13}C NMR and ^{1}H NMR techniques and identified bioactive compound chemical structure and name as 2-(3,4-dihydroxyphenyl)-3,4,4a,5-dihydro-2H-chromene-3,5,7-triol. This bioactive compound effectively scavenges and reduces various free radicals through the *in-vitro* approach. Hence, this bioactive compound may be considered a precursor molecule to prepare antioxidant agents. However, *in-vivo* studies are needed to perform in the coming days to validate their maximum potential.

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Graphical abstract