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Research Article

PROMISING ROLE OF LUTEOLIN FROM THE SPROUTS OF COCOS NUCIFERA L. IN INDOMETHACIN-INDUCED GASTRIC ULCER OF HUMAN GASTRIC ADENOCARCINOMA CELL LINE

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ABSTRACT

Plant-derived secondary metabolites and its derivatives represents the richness of potent novel bioactive compounds. Luteolin, a promising flavone belonging to flavonoids is present in fruits, vegetables and several medicinal plant and tree species with multiple pharmaceutical properties such as antioxidant, antiviral, diuretic, anti-inflammatory, anti-microbial, anticancer, anti-ulcer, antispasmodic, anti-allergic, anti-secretory, anti-angiogenic, anti-proliferative and several other properties. *Cocos nucifera* L. (Family: Arecaceae) is a well-known tree species commonly known as 'Kalpavriksha'. The aim and objective of the study is to isolate a promising anti-ulcer bioactive compound, its characterization, bioassays and to test its anti-ulcer activity using an *in vitro* cell line model. Separation, isolation and purification of a promising bioactive compound from the coconut sprouts was performed through silica gel column and thin layer chromatography. The isolated fraction was then subjected to characterization by Ultra Violet-Visible (UV-Vis), Fourier Transform Infrared spectrophotometer (FTIR) analysis and structure elucidation through Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) studies. Based on the spectral details, the isolated bioactive compound from the sprouts of *Cocos nucifera* L. was identified as luteolin, a flavone compound. *In vitro* bioassays namely antioxidant assays (hydrogen peroxide assay, superoxide anion scavenging assay, metal chelating activity, $ABTS^+$ assay) and anti-inflammatory assays (cyclooxygenase-COX-1, COX-2 and 5-lipoxygenase- LOX inhibitory activity) proved the bioactive potential of luteolin with minimum IC_{50} values. Anti-ulcer activity of luteolin was confirmed through its cytotoxic effect against human gastric adenocarcinoma epithelial (AGS) cell line with indomethacin-induced gastric ulcer which indicated a maximum per cent inhibition of infected AGS cells (90.39±0.26) with an IC_{50} value of 30.09 µg mL^{-1} whereas cimetidine (standard anti-ulcer drug) had 94.85±0.29 per cent inhibition with an IC_{50} value of 18.83 µg mL^{-1}. In the present work, identification of luteolin, a flavone has been reported for the first time from the sprouts (haustorium) of *Cocos nucifera* L. which is considered to be a promising anti-ulcer agent.

Keywords: Coconut sprouts, luteolin, flavone, anti-ulcer, AGS cell line.

1. INTRODUCTION

Cocos nucifera L. (Family: Arecaceae), the coconut palm (Kalpavriksha) is a well-known promising monocot, perennnial tree species considered to be enriched with medicinal and pharmaceutical properties. Generally, the coconut palms are grown throughout the world especially in tropics and are abundantly found in India, Australia, Indonesia, South America, Africa, Southeast Asia and Pacific Islands [1]. About 90 per cent of the coconut production in India is from Tamil Nadu, Kerala, Andhra Pradesh and Karnataka [2]. The coconut palm is considered as a versatile tree species for its multi-utilization in nutrition, medicine, food, pharmaceutical, agricultural, cosmetics, culinary and various other applications. Depending on the fruiting, coconut palms are classified into two types namely, tall and dwarf. It is reported that the tall varieties are considered as slow growers and dwarf varieties are fast growers [3]. The pharmaceutical properties such as anti-inflammatory, antimicrobial, antioxidant, anti-neo-plastic, anti-parasitic, anti-leishmania, anti-convulsant, antimalarial, antidiabetic and antiprotozoal activities of

coconut palm and its products are well documented. It is reported to exhibit antidote, cardio protective, antithrombotic, anti-artherosclerotic, hypolipidemic, anticholecystitic, immune stimulatory and hepatoprotective effects [4].

After the germination of the coconut, the embryo (basal part) embedded in the solid endosperm found near the germinating pore enlarges to form a cotyledon known as 'sprout' or 'haustorium'. The cardioprotective effect of the sprouts of *Cocos nucifera* L. was evaluated and the coconut sprouts are reported to be beneficial in the protection of heart diseases during myocardial infarction [5]. Coconut sprouts wine with antioxidant activity was produced using *Saccharomyces cerevisiae* [6]. From Gas-Chromatography-Mass Spectrometry (GC-MS) analysis, squalene, a potent triterpenoid with anti-ulcer property was reported in the sprouts of *Cocos nucifera* L. through *in silico* docking [7]. However, there is no study related to the isolation of bioactive compounds from the sprouts of *Cocos nucifera* L.

Peptic ulcers are acid induced severe lesions in the digestive tract which is usually characterized by a denuded mucosa with a defect usually extend upto muscularis propria or submucosa. It is reported that 5 to 10 per cent of total population is affected with peptic ulcer [8]. Gastric and duodenal ulcers (peptic ulcers) occur, i) due to non-steroidal anti-inflammatory drugs, ii) due to infection caused by *Helicobacter pylori*, a peptic ulcer causing gram-negative bacterial strain, iii) due to enormous acid peptic hypersecretion particularly in Zollinger-Ellison syndrome [9]. Several reports have revealed that functional polymorphisms in various cytokine genes are often related with peptic ulcers. Continuous usage of non-steroidal anti-inflammatory drugs with serotonin reuptake inhibitors, anticoagulants and corticosteroids increase the bleeding in upper gastrointestinal layer. However, epithelial cells degeneration and injury are caused by *Helicobacter pylori* by the inflammatory response with lymphocytes, macrophages, plasma cells and neutrophils. There are several anti-ulcer drugs which act as proton-pump inhibitors (omeprazole, rabeprazole, lansoprazole, pantoprazole, esomeprazole), antacids (magnesium hydroxide and aluminum hydroxide), cytoprotective agents (sucralfate, misoprostol), H_2 receptor blockers (famotidine, cimetidine, ranitidine, nizatidine) and potassium-competitive acid blockers (vonoprazan). However, all these modern commercial anti-ulcer drugs cause adverse side effects such as headache, diarrhea, abdominal pain, nausea, osteoporosis, depression, constipation, upper respiratory tract inflammation, cardiovascular problems and many other diseases [8]. Hence, identifying a novel drug without any side effects from a natural product is the need of the hour for the prevention and management of peptic ulcers.

Different types of secondary metabolites namely, alkaloids, phenols, tannins, flavonoids, terpenoids, phytosterols, glycosides and many other are reported in medicinal plants. Bioactive flavonoids are a major class of secondary metabolites in plants and plant-derived food products with promising pharmaceutical properties. It is reported that flavonoids i) exert potent anti-ulcer effects through the regulation of gastric secretion pathways, ii) exhibit gastric cytoprotective effect by the regulation of prostaglandin level, iii) act as a potent antioxidant agent and iv) possess anti *Helicobacter pylori* activity [10].

The sprouts (haustorium) of *Cocos nucifera* L., a phyto-medicine is a boon to the pharmaceutical industries for novel drug development to prevent and cure peptic ulcers specially gastric ulcers which is predominant worldwide. The coconut sprouts can also be recommended for food industries as nutraceutical product with therapeutic applications. Since not much work has been carried out in the sprouts (haustorium) of *Cocos nucifera* L., the aim and objective of the study is to isolate a promising anti-ulcer bioactive compound, its characterization, bioassays and to test its anti-ulcer activity using an *in vitro* cell line model.

2. MATERIAL AND METHODS

2.1. Collection of samples

Sprouts/haustorium of *Cocos nucifera* L. were collected from Thirunelveli District, Tamil Nadu, India. Coconut sprouts were separated from their respective shell, sliced into small pieces, dried using shade dry method (3 weeks). It was then powdered with a sterile mortar and pestle, stored in air tight glass containers (-4°C) for the present research work [7] (Fig. 1).

2.2. Preparation of the crude extract

In our earlier study [7], as there were no marked variations observed between the methanol and aqueous crude extracts with respect to the quantification studies, bioassays and characterization studies, the crude methanol extract of the sprouts (haustorium) of *Cocos nucifera* L. was subjected for the separation/isolation and characterization of a specific bioactive compound, it's *in vitro* bioassays and its application through cell line study. Cold percolation method (1:10 w/v) was

followed for the crude extract preparation using methanol as solvent [11]. It was filtered using Whatman No. 1 filter paper, concentrated at 30°C and the dried solvent-free crude powder of the sample was collected and stored in a glass container.

Fig. 1: (a) Sprouts (haustorium) of *Cocos nucifera* L. (b) Sliced pieces of the coconut sprouts/haustorium (c) Dried powder of the coconut sprouts/haustorium

2.3. Silica gel column chromatography and Thin Layer Chromatography (TLC) analysis

The collected dried solvent-free crude powder of the sample was subjected to silica gel column chromatography (60-120 mesh). For the preparation of admixture, to 10 g of the dried solvent-free crude powder of the sample, 30 g of pure silica gel was added and mixed thoroughly. The prepared admixture was loaded on a silica gel- column. Elution was then carried out using the solvent system (v/v), toluene: diethyl ether: acetic acid in different ratio- 100:0:0, 0:100:0, 0:0:100, 90:05:05, 80:10:10, 70:20:10, 60:30:10, 60:20:20, 50:30:20 and 50:25:25.The collected eluted samples were spotted on the TLC sheets (Kieselgel 60 F_{254}) and the characteristic coloured spots were visualized using Natural Product/Poly Ethylene Glycol (NP/PEG) spraying solution [12]. From the TLC analysis, the eluents used in different ratio was pooled into 1 to 70 fractions as follows,

Toluene: diethyl ether: acetic acid (100:0:0) - Fraction 1 to 6

Toluene: diethyl ether: acetic acid (0:100:0) - Fraction 7 to 11

Toluene: diethyl ether: acetic acid (0:0:100) - Fraction 12 to 15

Toluene: diethyl ether: acetic acid (90:05:05) - Fraction 16 to 20

Toluene: diethyl ether: acetic acid (80:10:10) - Fraction 21 to 27

Toluene: diethyl ether: acetic acid (70:20:10) - Fraction 28 to 36

Toluene: diethyl ether: acetic acid (60:30:10) - Fraction 37 to 51

Toluene: diethyl ether: acetic acid (60:20:20) - Fraction 52 to 58

Toluene: diethyl ether: acetic acid (50:30:20) - Fraction 59 to 64

Toluene: diethyl ether: acetic acid (50:25:25) - Fraction 65 to 70

The above set of 10 combined concentrated fractions were subjected to TLC analysis using the TLC sheets (Kieselgel 60 F_{254}) with its standard. The same developing solvent system (v/v), toluene: diethyl ether: acetic acid in a standardized ratio- 100:0:0, 0:100:0, 0:0:100, 90:05:05, 80:10:10, 70:20:10, 60:30:10, 60:20:20, 50:30:20 and 50:25:25 were used. The characteristic coloured spots were then visualized using NP/PEG spraying solution. The 10 different combined fractions having the similar characteristic coloured spots and R_f value were pooled together to obtain a single final fraction. For final confirmation of single fraction, TLC analysis was repeated using TLC sheets (Kieselgel 60 F_{254}) with its respective standard. The standardized solvent system, Toluene: diethyl ether: acetic acid-60:30:10 (v/v) was used and the characteristic spots were visualized using NP/PEG spraying solution [12]. Further, the final fraction of the separated bioactive compound was taken for characterization studies.

2.4. Characterization and spectral studies of the isolated bioactive compound

2.4.1. Ultra Violet-Visible (UV-Vis) Spectrophotometer analysis

The stock solution was prepared by dissolving 1 mg of the final fraction of the isolated bioactive compound in 1 mL of methanol. 1 mL of the stock solution was

analyzed for the presence of secondary metabolites based on the absorbance values using a UV-Vis Spectrophotometer (UV-1650PC Shimadzu) with a range between 200-800 nm [13].

2.4.2. Fourier Transform Infrared Spectrophotometer (FTIR) analysis

Potassium bromide pellet technique was followed [14]. The stock solution was prepared by dissolving 2 mg of the final fraction of the isolated bioactive compound in 2 mL of methanol. 2 mL of the stock solution was thoroughly evaporated using a flash evaporator and thin pellets were made using potassium bromide salt (1:100). FTIR spectral system (Shimadzu, IR affinity 1, Japan) with Deuterated Lanthanum α Alanine doped Tri Glycine Sulphate (DLaTGS) detector having a scan range of 400-4000 cm^{-1} with a specific resolution (4 cm^{-1}) and a mirror speed (2.8 mm sec^{-1}) was used for the study. Further, the infrared (IR) spectra were recorded (4000-500 cm^{-1}) [15, 16].

2.4.3. Nuclear Magnetic Resonance (NMR) studies and Mass Spectrometry analysis

NMR studies (^{1}H and ^{13}C) and mass spectrometry analysis of the isolated bioactive compound was conducted at Vellore Institute of Technology (VIT), Sophisticated Instrumentation Facility (SIF), School of Advanced Sciences, Chemistry Division, Vellore, Tamil Nadu, India. Stock solution was prepared by dissolving 1 mg of the final fraction of the isolated bioactive compound in 1 mL of 0.2 % Dimethyl Sulfoxide (DMSO). 1 mL of the stock solution was loaded into a FT-NMR BRUKER spectrometer to record the NMR data. The varied chemical shifts (ppm) were analyzed. After the separation by gas chromatography, Perkin-Elmer mass spectrometry model was used with a transfer line temperature and ion source temperature of 240°C. Further, the ionization mode electron impact was 70 eV with a particular scan time of 0.2 sec. The scan interval was 0.1 sec- fragments from 40 to 600 Da. The isolated bioactive compound from the sprouts of *Cocos nucifera* L. was characterized.

2.5. Structure elucidation of the isolated bioactive compound

Based on the UV-Vis spectrophotometer, FTIR analysis, NMR and MS studies, the two dimensional structure of the isolated bioactive compound was drawn using the software, ChemDraw Ultra version 12.0.

2.6. *In vitro* bioassays of the isolated bioactive compound

The stock solution was prepared by dissolving 0.01 g of the final fraction of the isolated bioactive compound in 10 mL of methanol. Different concentrations (20 µg mL^{-1}, 40 µg mL^{-1}, 60 µg mL^{-1}, 80 µg mL^{-1} and 100 µg mL^{-1}) were prepared and *in vitro* bioassays were conducted. Triplicates were maintained.

2.6.1. In vitro antioxidant assays

Various *in vitro* antioxidant assays related to gastric ulcer such as hydrogen peroxide assay [7], superoxide anion scavenging assay [17], determination of metal chelating activity [18] and 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ABTS assay [19] were carried out.

2.6.2. In vitro anti-inflammatory assays

Cyclooxygenase (COX-1 and COX-2) [20] and 5-lipoxygenase (LOX) inhibitory activity [21] were performed to determine the anti-inflammatory potential of the isolated bioactive compound.

2.7. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] cytotoxic assay

Human gastric adenocarcinoma (AGS) cell line with mucus-secreting epithelial cells were procured from National Centre for Cell Science (NCCS), Pune, India. These cells were maintained in Ham's F-12 essential media containing 10 % Fetal Bovine Serum, 100 µg/mL penicillin, 100 µg mL^{-1} streptomycin, 15 mM Hepes buffering solution and 1.2 g L^{-1} sodium bicarbonate incubated at 5 % carbon-di oxide (CO_2) incubator (37°C). The cells were then 100 per cent infected with gastric ulcer by exposing the cells for one hour to 10 mM indomethacin, a non-steroidal anti-inflammatory drug [22]. AGS infected cells (5,000 cells per well) loaded in a sterile 96 well plate were incubated in the culture medium with the stock solution of isolated bioactive compound (20 µg mL^{-1}, 40 µg mL^{-1}, 60 µg mL^{-1}, 80 µg mL^{-1} and 100 µg mL^{-1}) individually. It was then incubated at 37°C for 24 hours at 5 % CO_2 incubator. Then, 50 µL of freshly prepared MTT solution was added and incubated at 37°C for 4 hrs. Supernatant was discarded from the wells and 100 µL acidic isopropanol (0.04 M of HCl in isopropanol) was added and incubated overnight (room temperature). Absorbance was read at 570 nm using a microplate reader. Cimetidine, an anti-ulcer commercial drug was used as reference drug. Percent AGS infected cell inhibition and IC_{50} values were calculated [23].

Per cent cell inhibition = Abs Control- Abs Test/ Abs Control × 100

2.8. Statistical analysis

For each experiment, data presented are the means of three replicates. Values are expressed as mean ± SE of three replicates.

3. RESULTS

3.1. Silica gel column chromatography and Thin Layer Chromatography (TLC) analysis

Methanol extract of the sprouts (haustorium) of *Cocos nucifera* L. was eluted by silica gel column chromatography using the solvent system, Toluene: diethyl ether: acetic acid (v/v) at different ratio. The presence of an anti-ulcer bioactive compound was evaluated in each fraction by TLC analysis. A total of ten major combined fractions were obtained from the methanol extract of the sprouts (haustorium) of *Cocos nucifera* L. Ten different concentrated fractions evaluated by TLC analysis with its respective standard, from which the pooled fractions IV (fractions 16-20), VI (fractions 28-36), VII (fractions 37-51), IX (fractions 59-64) revealed similar characteristic coloured spots and R_f value. These fractions were then pooled together to obtain a single final fraction which had yield of 2.7 g. TLC analysis was performed for this single final fraction and the presence of a yellow bioactive compound (R_f value 0.31) was detected.

3.2. Characterization and spectral studies of the isolated bioactive compound

3.2.1. Ultra Violet-Visible (UV-Vis) Spectrophotometer analysis

In UV-Vis spectrophotometric analysis, the presence of flavonoids in the methanol extract of the sprouts (haustorium) of *Cocos nucifera* L. was detected at a range from 200-400 nm (Fig. 2). UV-Visible spectra indicated strong bands (355.00 nm, 338.50 nm, 257.50 nm, and 224.50 nm) with no specific decrease in the intensity which is a characteristic feature of flavones (Table 1).

Table 1: UV-Vis analysis of the isolated bioactive compound

Band Number	Wavelength	Absorbance
1	355.00	3.101
2	338.50	2.872
3	257.50	2.960
4	224.50	2.834

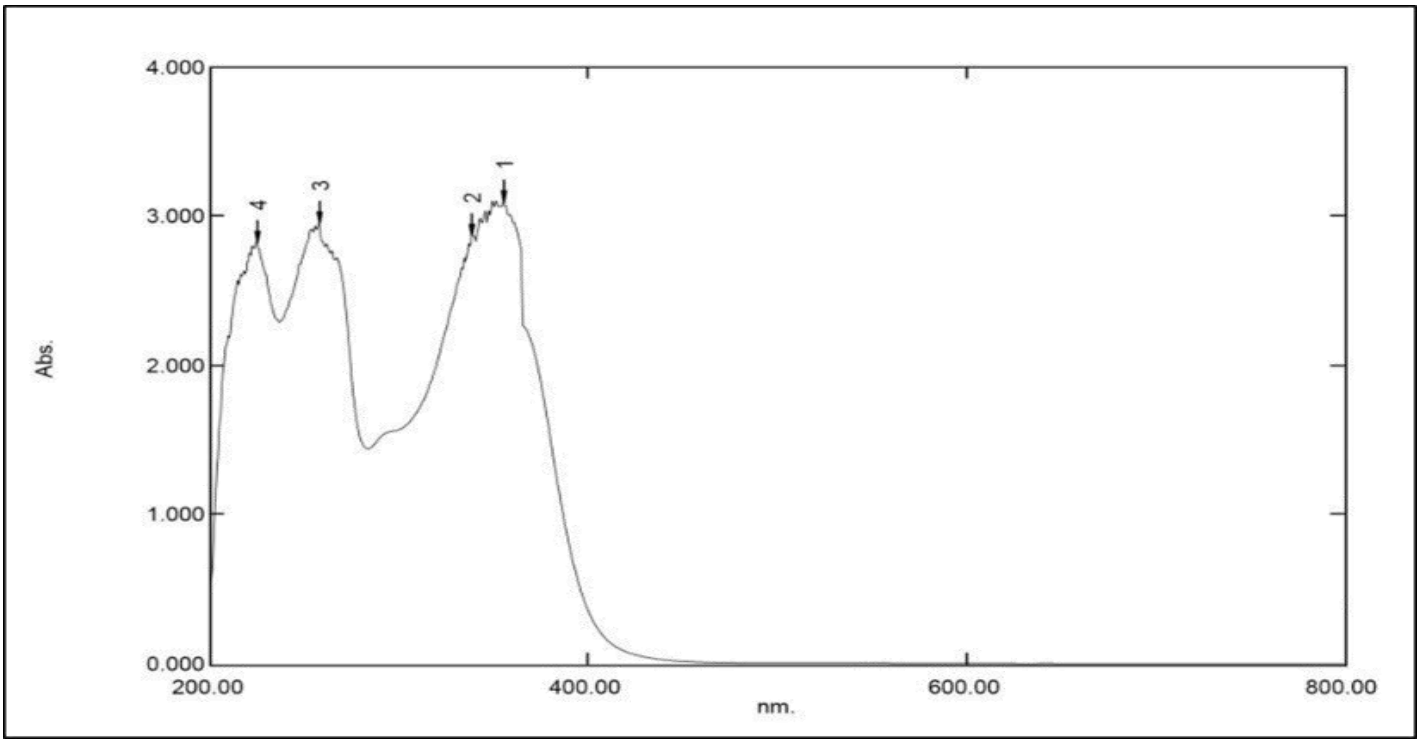

Fig. 2: UV-Visible spectrum of the isolated bioactive compound from the methanol extract of the sprouts (haustorium) of *Cocos nucifera* L.

3.2.2. Fourier Transform Infrared Spectrophotometer (FTIR) analysis

FTIR analysis of the isolated bioactive compound revealed several absorption peaks belonging to varied functional groups. However, the presence of a broad band at 3404.36 cm^{-1} corresponds to the stretching vibration of phenols with hydrogen bond. A weak band at 2926.01 cm^{-1} reveals C-H stretching vibration of aromatic group. The presence of a medium band at 2351.23 cm^{-1} corresponds to the C-H bending vibration of aliphatic group. A weak band at 2858.51 cm^{-1} represents the C-H stretching aliphatic group. Occurrence of a strong band at 1653 cm^{-1} reveals the C=O stretching vibration of aromatic group. A medium band at 1597.06 cm^{-1} represents the C=C stretching vibration of aromatic group (Fig. 3). These functional groups which were characterized are considered to be the characteristics of flavones.

3.2.3. Nuclear Magnetic Resonance (NMR) studies

Nuclear Magnetic Resonance studies (^{1}H and ^{13}C) revealed the strong bond connectivity of the isolated bioactive compound (Table 2). Moreover, it depicted the aglycone signal patterns. All these spectral details indicated the characteristic features of flavones belonging to the class of flavonoids (Figs. 4 and 5).

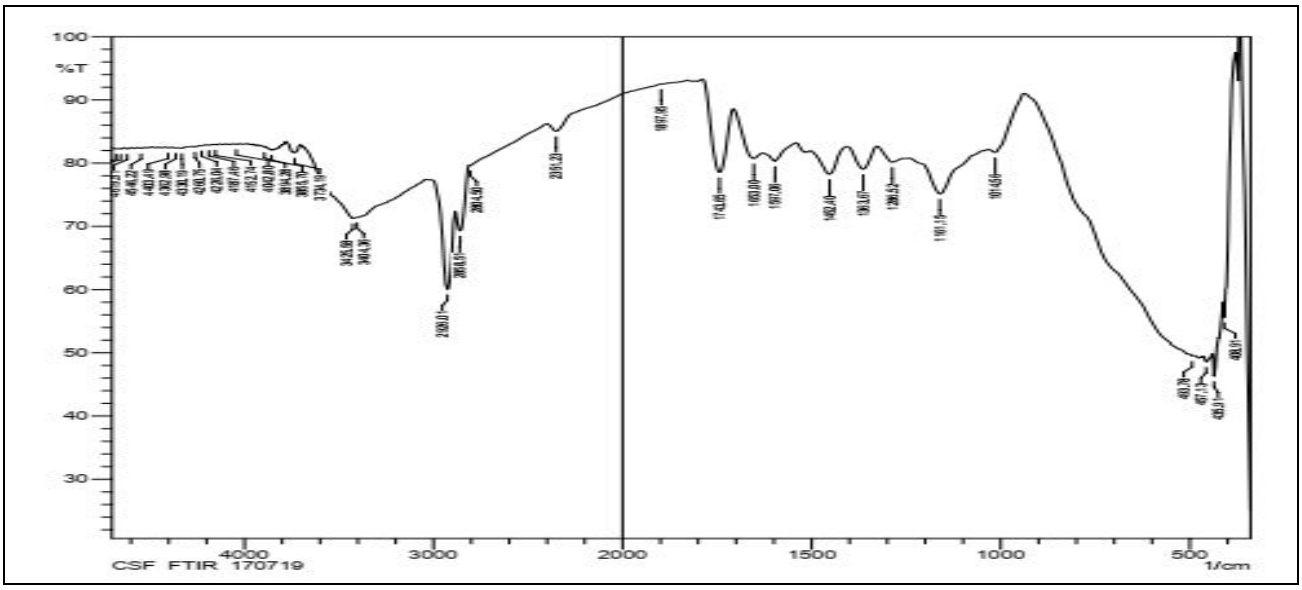

Fig. 3: FTIR spectrum of the isolated bioactive compound from the methanol extract of the sprouts (haustorium) of *Cocos nucifera* L.

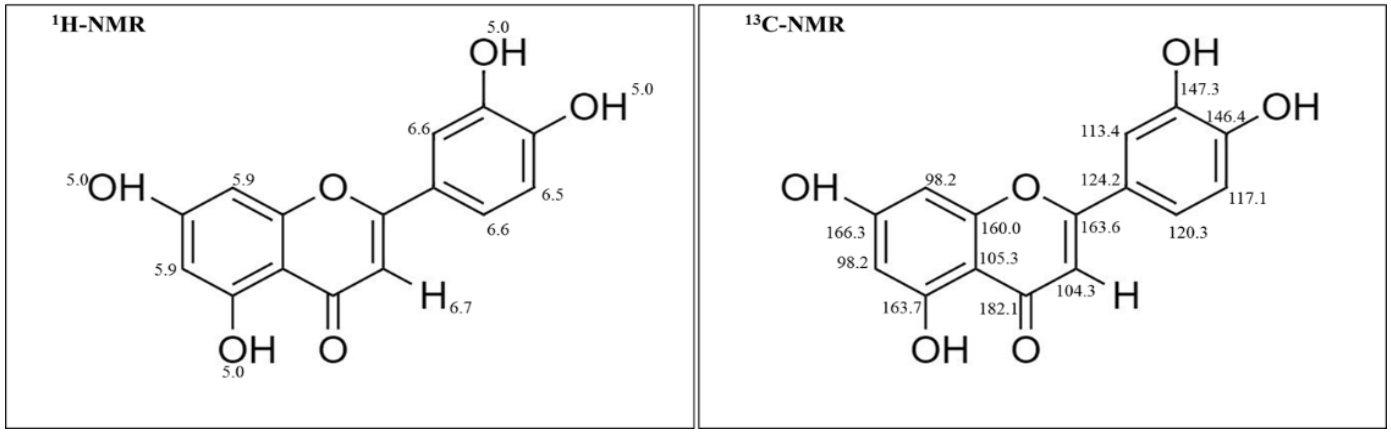

Fig. 4: Illustration of ^{1}H-NMR and ^{13}C-NMR peak data values of the isolated bioactive compound from the methanol extract of the sprouts (haustorium) of *Cocos nucifera* L.

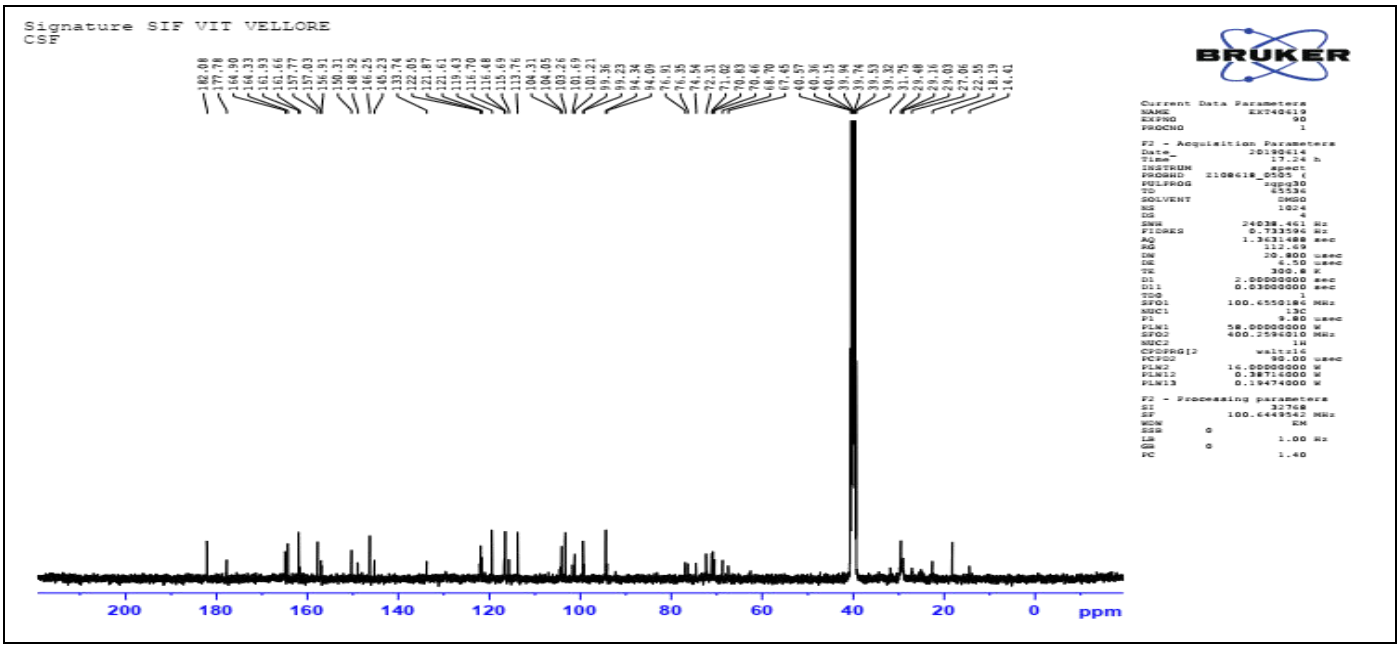

Fig. 5: NMR spectrum of the isolated bioactive compound from the methanol extract of the sprouts (haustorium) of *Cocos nucifera* L.

Table 2: NMR data of the isolated bioactive compound

S. No.	Type of NMR	NMR Data
1	^{1}H-NMR	5.9 (1H,s,H)1) 5.9 (1H,sH-3) 5.0 (1H,-OH) 6.7 (1H,s,H-6) 6.6 (1H,s,H-2) 6.5 (1H,s,H-31) 6.6 (1H,s,H-6^{1})
2	^{13}C-NMR	98.2 (C 1) 166.3 (C 2) 98.2 (C 3) 163.7 (C 4) 105.3 (C 4a) 182.1 (C 5) 104.3 (C 6) 163.6 (C 7) 160.0 (C 7a) 124.2 (C 2^{1}) 117.1 (C 3^{1}) 146.4 (C 4) 147.3 (C 5^{1}) 113.4 (C 6^{1})

3.2.4. Mass Spectrometry analysis

Mass Spectrometry analysis revealed the molecular weight of 286.24 g mol^{-1} with the presence of a molecular ion peak at m/z 284.00 which is considered as the nearest value of luteolin, a flavone (Fig. 6).

3.3. Structure elucidation of the isolated pure bioactive compound

UV-Vis spectrophotometer, FTIR analysis, NMR and MS studies revealed the specific functional groups, varied chemical shifts and the molecular mass of the isolated bioactive compound. Based on the obtained spectral details, the isolated bioactive compound was identified as luteolin, a flavone compound belonging to the class of flavonoids (Table 3). Further, the two dimensional structure of the isolated bioactive compound drawn using the software, ChemDraw Ultra version 12.0 is depicted in Fig. 7.

3.4. *In vitro* bioassays of the isolated bioactive compound

The antioxidant activity (hydrogen peroxide assay, super oxide anion scavenging assay, metal chelating activity and ABTS assay) and anti-inflammatory potential [cyclooxygenase (COX-1 and COX-2) and 5-lipoxygenase (LOX) inhibitory activity] of the isolated bioactive compound, luteolin from the sprouts (haustorium) of *Cocos nucifera* L. was determined through the per cent inhibition and IC_{50} values. IC_{50} value is the concentration of the extract required for 50 per cent inhibition. When the IC_{50} value is minimum, it denotes the maximum antioxidant and anti-inflammatory potential of the isolated bioactive compound.

Table 3: Identification of the isolated pure bioactive compound, Luteolin

Name of the compound with Chemical Abstracts Service (CAS) number	Synonyms	Molecular formula	Molecular weight (g mol^{-1})	Nature of the isolated bioactive compound	Biological activities (Source: PubChem database and NIST library) [https://pubchem.ncbi.nlm.nih.gov/ & http://www.nist.gov/]
Luteolin (491-70-3)	3',4',5,7-Tetrahydroxyflavone; Digitoflavone; Flacitran; Flavopurpol	$C_{15}H_{10}O_6$	286.24	Flavone, a secondary metabolite belonging to the class of flavonoids and a nutraceutical	Possess multiple pharmaceutical properties such as antioxidant, antiviral, diuretic, anti-inflammatory, antimicrobial, anticancer, anti-ulcer, antispasmodic, anti-allergic, anti-secretory, anti-histaminic, bacteriostatic, anti-angiogenic, anti-proliferative and several other bioactivities (Pub Chem database and NIST library)

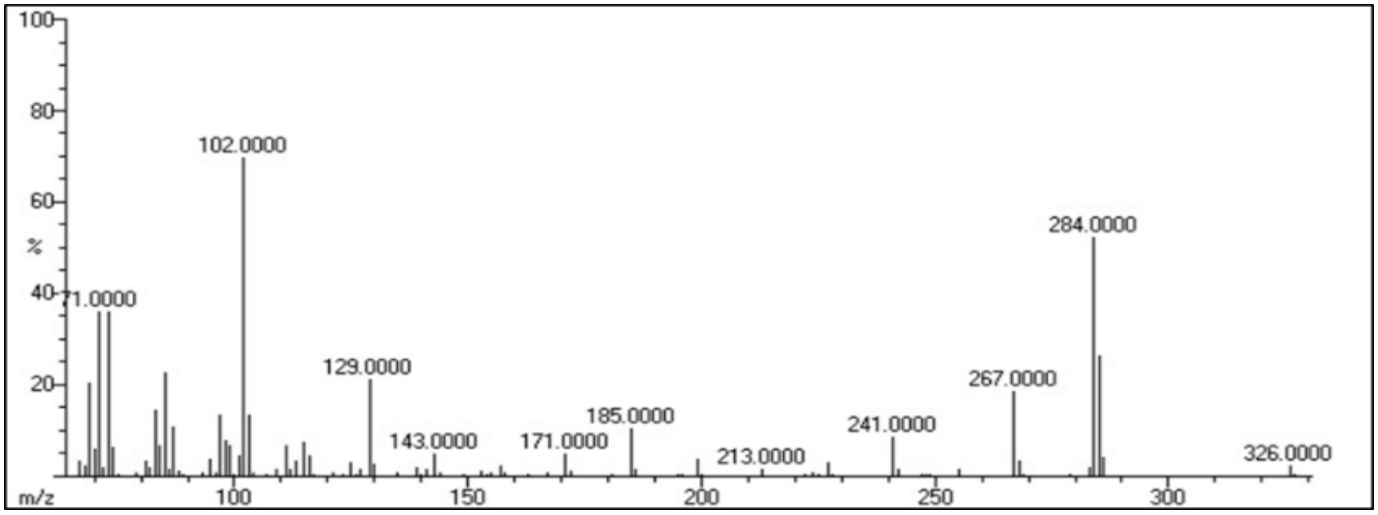

Fig. 6: Mass spectrum of the isolated bioactive compound from the methanol extract of the sprouts (haustorium) of *Cocos nucifera* L.

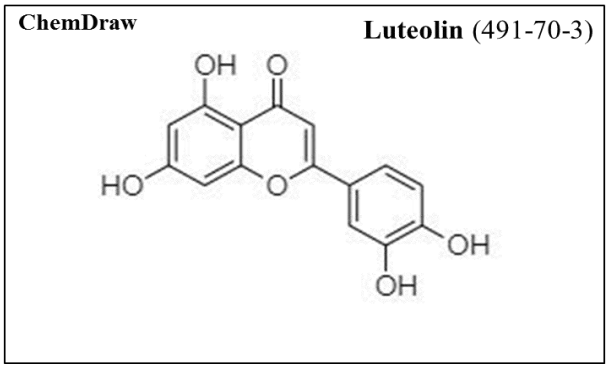

Fig. 7: Illustration of the structure of a bioactive compound Luteolin, a flavone isolated from the sprouts (haustorium) of *Cocos nucifera* L.

3.4.1. In vitro antioxidant assays

In hydrogen peroxide assay, luteolin (100 µg mL^{-1}) revealed maximum per cent inhibition of 75.25±0.29 with IC_{50} value of 51.30 µg mL^{-1} whereas the standard ascorbic acid at 100 µg mL^{-1} showed 81.12±0.28 per cent inhibition with IC_{50} value of 41.00 µg mL^{-1}. Superoxide anion scavenging assay indicated that luteolin (100 µg mL^{-1}) showed maximum per cent inhibition of 71.15±0.25 with IC_{50} value of 59.43 µg mL^{-1} whereas the standard ascorbic acid at 100 µg mL^{-1} had 77.72±0.28 per cent inhibition with an IC_{50} value 48.01 µg mL^{-1}.

In metal chelating activity, luteolin (100 µg mL^{-1}) revealed maximum per cent inhibition of 80.33±0.26

with IC_{50} value of 42.24 µg mL^{-1} whereas the standard Na_2EDTA at 100 µg mL^{-1} showed 86.24±0.26 per cent inhibition with IC_{50} value of 31.00 µg mL^{-1}. ABTS assay indicated that luteolin at 100 µg mL^{-1} showed maximum per cent inhibition of 83.42±0.26 with IC_{50} value of 39.56 µg mL^{-1} whereas the standard ascorbic acid at 100 µg mL^{-1} had 90.34±0.26 per cent inhibition with IC_{50} value of 28.68 µg mL^{-1}.

The isolated bioactive compound, luteolin revealed maximum potent antioxidant activity on par with the respective reference standard in *in vitro* antioxidant assays carried out related to gastric ulcer (Fig. 8).

3.4.2. In vitro anti-inflammatory assays

In cyclooxygenase (COX-1) inhibitory activity, luteolin at 100 µg mL^{-1} revealed a per cent inhibition of 54.46±0.27 with IC_{50} value of 89.83 µg mL^{-1} whereas the standard indomethacin at 100 µg mL^{-1} showed 87.22±0.27 per cent inhibition with IC_{50} value of 35.19 µg mL^{-1}. However, in cyclooxygenase (COX-2) inhibitory activity, luteolin at 100 µg mL^{-1} had maximum per cent inhibition of 83.97±0.25 with IC_{50} value of 39.94 µg mL^{-1} whereas the standard indomethacin at 100 µg mL^{-1} showed 90.42±0.25 per cent inhibition with IC_{50} value of 29.17 µg mL^{-1}.

5-lipoxygenase assay indicated that luteolin at 100 µg mL^{-1} showed maximum per cent inhibition of 87.84±0.29 with IC_{50} value of 34.07 µg mL^{-1} whereas the standard indomethacin at 100 µg mL^{-1} had 94.46±0.27 per cent inhibition with IC_{50} value of 21.85 µg mL^{-1}. Luteolin revealed maximum anti-inflammatory activity on par with the reference standard and the results are clear evidence that luteolin from the coconut sprouts can act as a selective COX-2 inhibitor (Fig. 9).

3.5. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] cytotoxic assay

Luteolin from the coconut sprouts (haustorium) at 100 µg mL^{-1} revealed maximum per cent inhibition of the infected AGS cells (90.39±0.26) with IC_{50} value of 30.09 µg mL^{-1}. However, cimetidine (standard anti-ulcer drug) at 100 µg mL^{-1} had 94.85±0.29 per cent inhibition with IC_{50} value of 18.83 µg mL^{-1}. Luteolin was found to be cytotoxic to the infected AGS cells with gastric ulcer in a dose dependent manner. An increase in the concentration of luteolin has increased the cytotoxic activity towards the infected AGS cells with gastric ulcer (Fig. 10). The purified isolated bioactive compound, luteolin from the sprouts of *Cocos nucifera* L. indicated maximum anti-ulcer activity on par with the reference standard commercial anti-ulcer drug (Fig. 11).

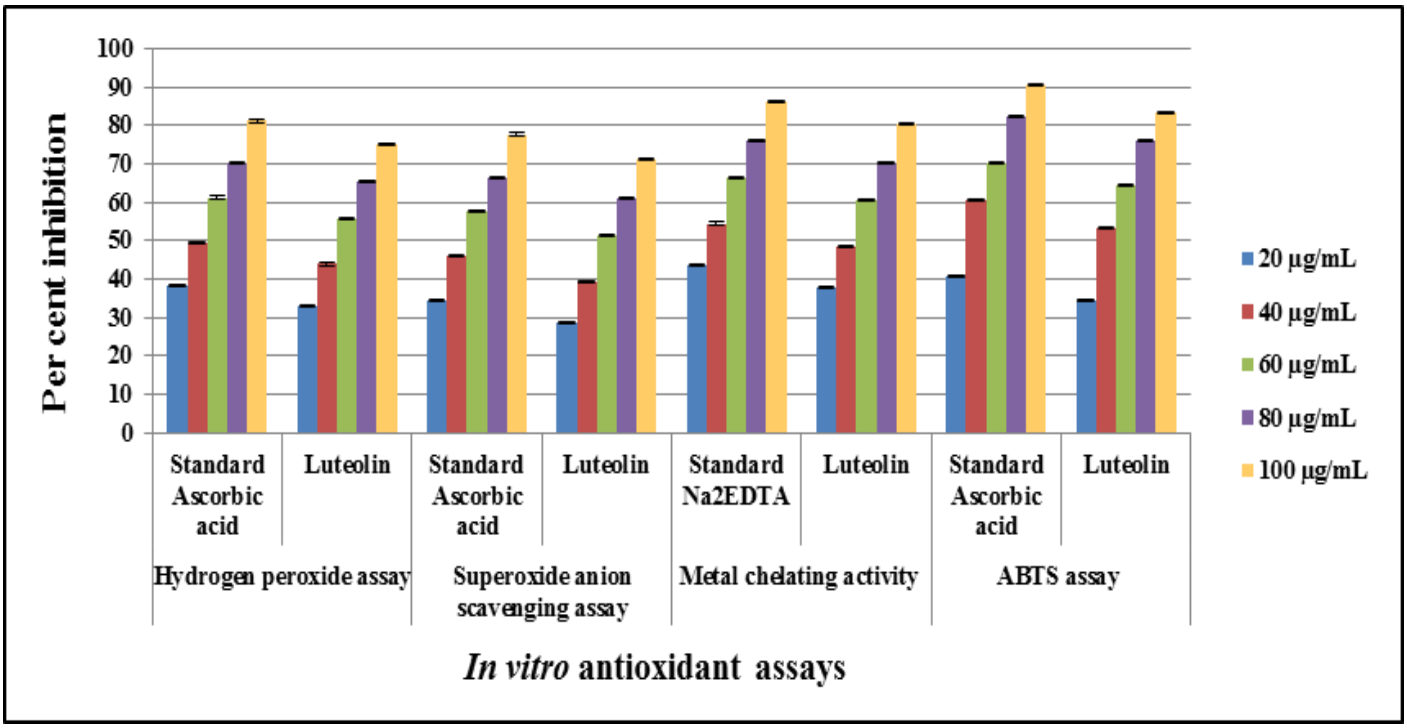

Fig. 8: *In vitro* antioxidant activity of luteolin from the sprouts (haustorium) of *Cocos nucifera* L. Values are expressed as mean ± SE (n= 3)

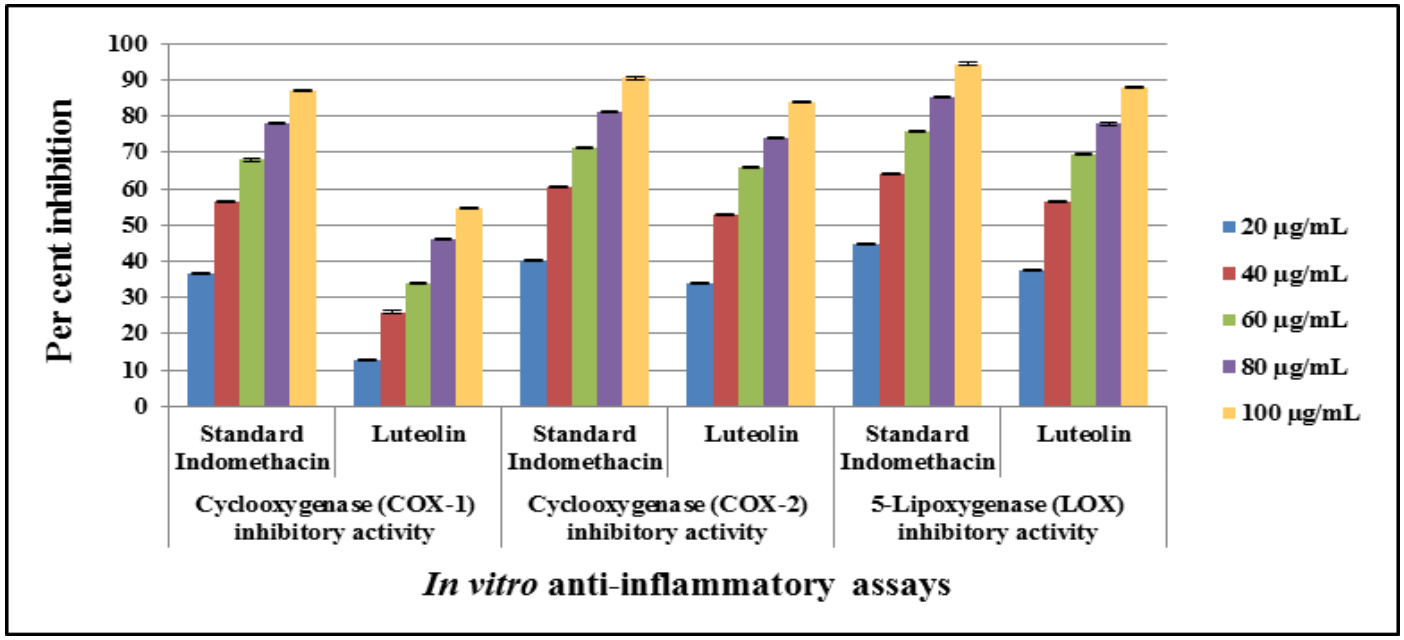

Fig. 9: *In vitro* anti-inflammatory activity of luteolin from the sprouts (haustorium) of *Cocos nucifera* L. Values are expressed as mean ± SE (n= 3)

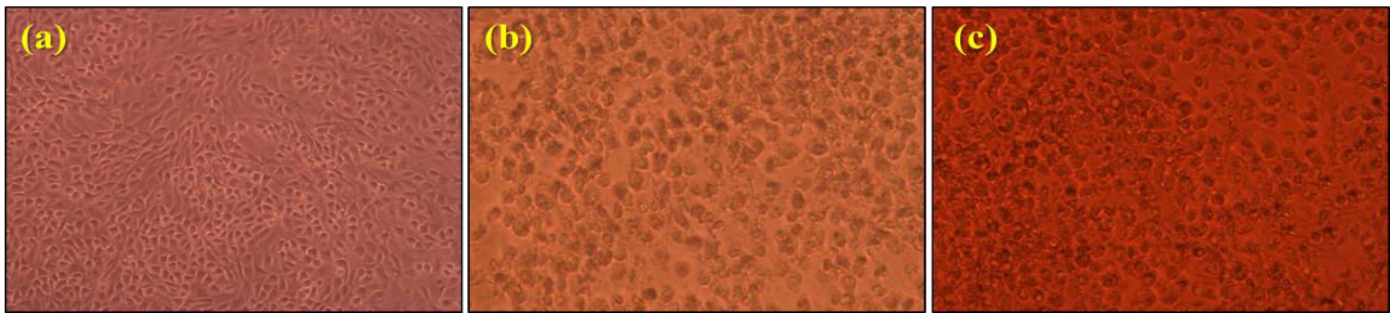

Fig. 10: Illustration of cytotoxic activity of standard and luteolin (100 µg mL^{-1}) against human gastric adenocarcinoma (AGS) mucus-secreting epithelial cell line with indomethacin- induced gastric ulcer (a) Control cells (b) Standard Cimetidine (c) Luteolin from sprouts (haustorium) of *Cocos nucifera* L.

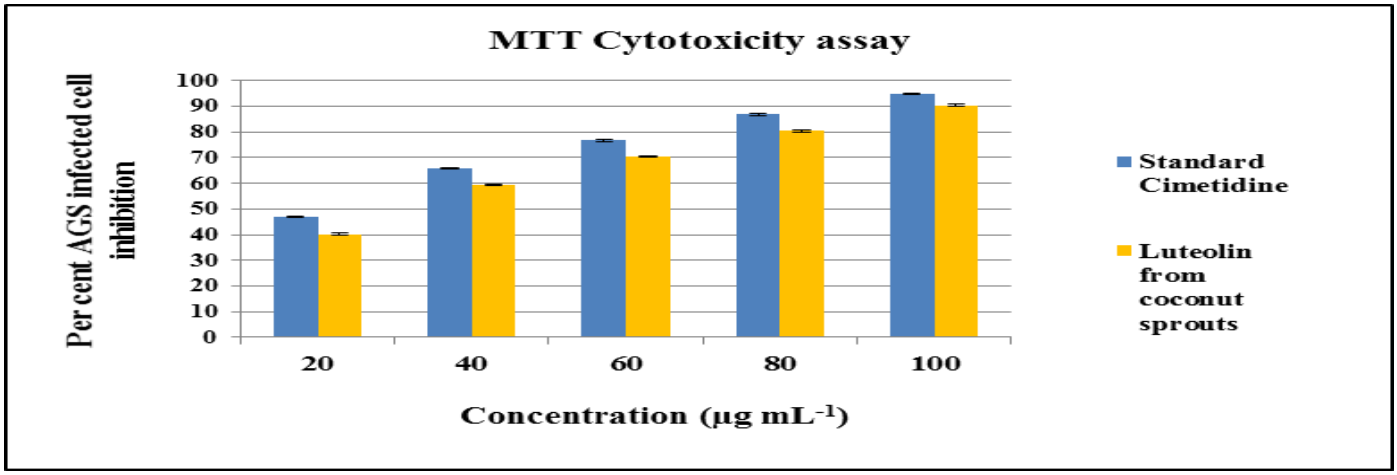

Fig. 11: Cytotoxic activity of luteolin from the sprouts (haustorium) of *Cocos nucifera* L. Values are expressed as mean ± SE (n= 3)

4. DISCUSSION

Natural products with numerous therapeutic applications are the major source of bioactive compounds which make them an important source of raw material for the development of medicines/novel drugs. In the present scenario, extensive investigation of the secondary metabolites in the plants and plant-derived natural food products is a need of the hour. One such promising tree species, *Cocos nucifera* L. (Kalpavriksha) yield various natural edible products which are used as a traditional medicine. Cold percolation method is reported to be an efficient method for the preparation of crude extracts of natural products as the saturated solvent will be replaced by the fresh solvent constantly [24]. Crude extract of the sprouts (haustorium) of *Cocos nucifera* L. which was freshly prepared was found to be very much suitable for the separation and isolation of the bioactive compound.

Chromatography is a technique for the separation, isolation, purification and identification of bioactive components from crude extracts or any type of complex mixture. Thin Layer Chromatography play an important role during the initial stages of identification of novel drugs and drug development [25]. Based on the formation of bands or spots, accurate standardization of the solvents were possible for the crude extract of sprouts (haustorium) of *Cocos nucifera* L. Silica gel column chromatography is an efficient and cost-effective separation method which usually provides high resolution when compared to other chromatographic purification methods. The major advantage is the usage of different types of solvents based on polarity [25]. The sprouts (haustorium) of *Cocos nucifera* L. is considered to be a complex mixture of bioactive compounds with essential fatty acids. However, the isolation of a characteristic flavonoid group of bioactive compound luteolin from the sample was found to be simple and accurate through the usage of silica gel column chromatography. Similar studies were carried out in the *Feronia limonia* fruit pulp [26].

Characterization of complex and pure mixtures are performed by physical methods generally. Besides various other techniques namely determination of density, melting point and refractive index, ultra violet-visible light spectroscopy is used in different fields of research, production and quality control for the drug classification and its distribution [27]. Strong bands with no specific decrease in the intensity observed in the isolated bioactive compound might be due to the presence of flavones. Fourier Transform Infrared Spectrophotometer (FTIR) analysis indicates the distribution of varied functional groups within the organic fractions [7]. Using FTIR, the structure of a functional group of a complex mixture can be accurately determined. It is a rapid technique to fingerprint the important characteristic features of any type of biological or complex sample under particular conditions [28]. Functional groups are usually related with IR absorption bands corresponding to major vibrations [29]. The presence of numerous bands (broad band, weak band, medium band) in the isolated bioactive compound corresponds to the stretches (functional groups) depicting the presence of luteolin. In general, NMR spectroscopy is widely used in the investigation of novel compounds which are of significant importance namely ceramics, synthetic membranes, glasses, polymers and superconductors. In food science and natural product research, NMR is used to check the authenticity of bioactive compounds and their identification. Area of an absorption peak is usually proportional to the number of nuclei with similar or same chemical shift. Thus, in ^{1}H-NMR, the chemical shifts are defined as the difference in the resonance frequencies between the nucleus of interest and a reference nucleus. Chemical shifts of ^{13}C are similar to ^{1}H and are measured in parts per million (ppm), ranging from 0 to 250 ppm [30]. NMR data recorded for the sample corresponds to luteolin, a flavone in the sprouts (haustorium) of *Cocos nucifera* L. Though NMR is an expensive method, it is considered as an accurate technique in the identification of bioactive compounds from natural crude extracts. Mass spectrometry analysis play a major role in structure elucidation of a bioactive compound. Separation of ions occur in the ionization source of mass spectrometer based on m/z ratios (mass-to-charge). The quasi-molecular ion peak observed in the sample indicated luteolin. Similar observations were reported in the fruit pulp of *Feronia limonia* [26].

Luteolin is considered to be a flavone with B-ring occurring in vegetables, fruits, plants with utmost medicinal value. After entering the human body, small intestine rapidly absorbs luteolin. It undergoes a metabolism by conjugation to monoglucuronides and diglucuronides by an enzyme, UDP-glucuronosyl-transferase [31]. Lipopolysaccharide (LPS) or endotoxin which is usually present in outer membrane of toxic gram-negative bacteria (*Helicobacter pylori*) is responsible for the severe infection which induce the release of several inflammatory toxic mediators [32]. It is reported that the luteolin can prevent LPS induced increased

inflammation in mRNA expression of IL-1β, Ccl2, Ccl3, Ccl5, IL-6 and NF-κB1 [31]. *Helicobacter pylori* infection, consumption of NSAIDs (non-steroidal anti-inflammatory drugs) and stress are considered to be the source for the recurrence of peptic ulcer. *H. pylori* infection and NSAIDs produce severe neutrophil infiltration into the mucosa through the stimulation of several inflammatory responses such as expression of toxic inflammatory cytokines namely interleukins and tumor necrosis factor. Severe injuries will be produced in the gastric mucosa. Gastric mucosal damage also occur due to stress which results in over expression of adhesion molecules, tumor necrosis factor and neutrophil infiltration. Inflammation in the mucosal layer is responsible for the severe damage of gastric mucosa resulting in peptic ulcers (gastric and duodenal ulcers) [33]. Promising role or mechanism of action of luteolin, a promising flavone belonging to flavonoids include suppression of oxidative stress, severe inflammation, neutrophil infiltration, apoptosis (programmed cell death) and induce gastroprotective activity by increasing the prostaglandin E_2synthesis, Hsp70 protein levels and synthesize gastric mucin [34].Coconut sprouts which are the natural source of luteolin can be used as a natural therapeutic agent.

Antioxidant molecules suppress the ROS (reactive oxygen species) formation through the oxidant enzyme inhibition or through chelation of metals or trace elements which are considered as the source for the generation of free radicals. These natural antioxidant molecules augment the bioactivities of antioxidant enzymes [35]. Promising antioxidant activity was shown by luteolin from the sprouts (haustorium) of *Cocos nucifera* L. Hydrogen peroxide, a weak oxidizing agent deactivate some of the enzymes by oxidation of thiol groups. Formation of toxic hydroxyl radicals occur through the reaction of hydrogen peroxide with Fe^{2+} and Cu^{2+} ions [7]. Removal of hydrogen peroxide is essential for the antioxidant defense in a cell system [35]. ROS are reported to play an important role in gastro-intestinal tract through ischemia-reperfusion and several necrotizing agents like hydrogen peroxide which is toxic to cells at increased levels [36]. Luteolin from the coconut sprouts thoroughly scavenged the hydrogen peroxide which is due to its potent activity of neutralizing hydrogen peroxide to water by donating electrons. This mechanism of action is reported in various phenolic and flavonoid compounds.

Superoxide anions or radicals induce lipid oxidation and act as precursor molecule for ROS generation [35]. Superoxide anions are formed when an electron is added to molecular oxygen. It induces oxidation reaction by reducing the transition metals to pro-oxidative form and by promoting release of metals specially from protein molecules [37]. Superoxide radicals are less reactive and the damage of cells have been attributed to HO which is produced through Haber-Weiss process (O^{2-}reduces Fe^{3+} to Fe^{2+} ions) [38]. Excessive production of free radicals and lipid peroxidation are solely responsible for the occurrence of several diseases particularly gastric ulcer [39]. Flavonone and dihydroflavonol possess a single bond between C2 and C3 whereas flavonols and flavones have a double bond between C2 and C3. This major difference in the structure influences the antioxidant activity reported in earlier studies. Double bond between C2 and C3 signifies that the B ring will usually be coplanar with A and C rings because of conjugation. When this double-bond saturation occurs, conjugation and the coplanarity will be terminated. This study suggested that planar structure of flavonoid is essential for antioxidant scavenging activity [40]. However, the present findings was found to be importance as luteolin indicated a promising superoxide anion scavenging activity proving that there is no correlation or potent effect because of the occurrence of double bond between the positions C2 and C3. Luteolin from the coconut sprouts scavenged superoxide radicals showing its promising antioxidant potential.

Ferrozine quantitatively chelate with Fe_2^+ and produce a red colour complex in metal chelating assay [41]. When any type of flavonoid interacts with a metal ion, it leads to the formation of a chelate complex which is essential in preventing the generation of free radicals causing oxidative damage was reported in the previous study. Usage of synthetic metal chelators as an antioxidant produces severe side effects [42]. Luteolin from coconut sprouts with prominent metal chelating property can act as a natural metal chelator by suppressing the free radical generation and inflammatory responses related to peptic ulcers. A natural antioxidant molecule scavenges $ABTS^+$ radical cations which causes severe oxidative stress. These radicals cause disruption of gastric mucosal layer leading to peptic ulcers [43]. In several studies it has been observed that the solubility of extracts or the stereoselectivity of radicals in various testing systems are tend to affect the nature of the extract or sample to scavenge free radicals [44]. The present study was found to be of utmost significance as

there was no dispute observed in such factors and luteolin revealed potent antioxidant activity as a free radical scavenger and a metal chelator protecting the gastric mucosa.

The mechanism of action of NSAIDs and *H. pylori* infection is by inhibiting the prostaglandin synthesis resulting in ulceration, gastric mucosa damage and several complications in the gastrointestinal tract. Prostaglandins act as a protective agent against several luminal insults such as bile salts, antigens and acids by maintaining the mucosal blood flow, bicarbonate, mucus secretion and protecting the epithelial and endothelial cells [45]. Moreover, prostanoids/prostaglandins thoroughly mediate human body's reactions or responses to inflammation and tissue injuries. COX enzymes are isoenzymes which are genetically independent proteins with different properties. Generally, COX-1 enzyme is expressed throughout the human body and constitutes gastrointestinal protection, however COX-2 enzyme is not present in normal tissues. It's expression is produced by the strong stimuli namely oncogenes, proinflammatory cytokines, mitogens, lipopolysaccharides and hormones resulting in prostaglandin synthesis in increased levels in neoplastic and inflamed tissues. COX-2 has been involved in pathological mechanism namely inflam-mation and cancers. Thus, the NSAIDs are considered to be potent competitive inhibitors of COX, an enzyme which is responsible in the bioconversion of arachidonic acid to toxic inflammatory prostaglandins. Anti-inflammatory action is caused by inhibiting COX-2, but side effects occur when the entire COX-1 is inhibited [46]. Luteolin from coconut sprouts (haustorium) was able to act as a potent selective COX-2 inhibitor thereby preventing inflammation which causes gastritis. The present findings coincide with a similar study which indicated that luteolin and other flavones exhibit promising anti-inflammatory activity [47].

Lipoxygenase enzymes are rich in non-heme iron and catalyze arachidonic acid/linoleic acid peroxidation with molecular oxygen. Over expression of LOX enzymes and leukotrienes are reported in gastrointestinal tract leading to gastric ulcers/cancers. Among several human LOXs, 5-LOX is considered as a major inflammation intermediate [48]. Flavonoids are reported to inhibit chronic/acute inflammation. Luteolin inhibited 5-LOX indicating its anti-inflammatory potential. Suppression of ROS and synthesis of eicosanoids due to the strong interference with LOX, COX and phospholipase A_2 enzymes may be luteolin's mechanism of action [49].

AGS cell line derived from human stomach adenocarcinoma with mucus-secreting epithelial cells was used in the present study as an *in vitro* model to determine the anti-ulcer activity of luteolin. The major reason of using this particular cell line and its advantages are, it retain the characteristics of normal gastric epithelial cells namely i) microvilli, ii) epithelial morphology, iii) mucus production. These are the typical characteristics which are essential in the studies related to ulcers [21]. MTT assay determines the mitochondrial functional state. Mitochondrial dehydrogenase in the living cells generally reduces MTT salt (yellow tetrazolium) to MTT blue formazan which gets precipitated in the uninjured cells [50]. Indomethacin, an indol derivative NSAID is the first choice of interest in producing gastric ulcer experimental model due to its strong ulcerogenic effect. It induces mucosal damage through the inhibition of several protective factors such as COX-1, PGE2, mucus and bicarbonate. It increases the acid levels and oxidant activities [51]. The present findings are clear evidence that luteolin from coconut sprouts is said to be non-toxic bioactive flavone with promising anti-ulcer activity. Moreover, an increase in concentration of the luteolin, increased its per cent inhibition of AGS infected cells. MTT assay proved that luteolin from coconut sprouts were observed to be cytotoxic to infected AGS cells with gastric ulcer in concentration dependent manner. Luteolin from coconut sprouts proved to be a selective COX-II inhibitor, thereby indicating that the flavone will not induce ulceration. It possesses potent antioxidant potential which can be correlated to its anti-ulcer property as it is a ROS scavenger and metal chelator. Standard cimetidine which was used in the present study is reported to possess potent anti-histaminic properties [52] which will usually reduce the excess secretion of HCl into gastric lumen. As luteolin possess these similar properties, it can be recommended instead of cimetidine as it is obtained from a natural plant-derived product.

Studies revealed that luteolin's vasodilatory effects had increased the submucosal blood flow which enables healing of ulcers by inhibiting protein kinase C and cyclic nucleotide phosphodiesterase. Luteolin is reported to have bacteriostatic property acting as a selective inhibitor of arylamine N-acetyltransferase in *Helicobacter pylori* [52]. Thus, luteolin can act as a strong anti-ulcer agent against peptic ulcers.

The novelty of the present study includes,

(i) identification of luteolin, a flavone-a promising anti-ulcer agent that has been reported for the first

time from the sprouts (haustorium) of *Cocos nucifera* L.;

(ii) coconut sprouts are the major source of luteolin which is cost-effective and can be used as a nutrient supplement, natural therapeutic agent without any side effects, further to be taken for the development of novel drugs with clinical trials;

(iii) Fresh and dried form of coconut sprouts with luteolin apart from an anti-ulcer agent can be further used for the prevention and management of other diseases as it is enriched with numerous essential bioactive compounds with pharmaceutical properties.

5. CONCLUSION

Luteolin, a promising flavone from the sprouts of *Cocos nucifera* L., was identified as a potent anti-ulcer agent. Luteolin is considered to be non-toxic as it is enriched with maximum bioactive potential with antioxidant, anti-inflammatory and anti-ulcer properties. Thus, the coconut sprouts are the major natural source of luteolin with a wide range of biological activities which can act against peptic ulcers in a cost-effective manner. Further, luteolin from coconut sprouts can be recommended to pharmaceutical industries for large scale drug development with quality check and clinical trials. Moreover, the coconut sprouts can be recommended for consumption as a nutrient supplement as the sprouts are enriched with a potent nutraceutical substance, luteolin.

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