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Evaluation of prebiotic potential and HPTLC analysis of *Glycyrrhiza glabra* with viewpoint of GI inflammation of chemotherapy

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ABSTRACT:
Cancer and its related harmful effects on treatment techniques, such as radiation and chemotherapy, greatly diminish the quality of life for individuals suffering from the disease. Consequently, methods to control side effects caused by radiation and chemotherapy have received more attention as of late. Glycyrrhiza glabra, also known as liquorice, is a perennial herb with a long history of use in traditional medicine. Scientists have studied its possible medicinal uses at length. Liquorice and related regimens taken at the same time as chemotherapy may lessen the side effects of the latter. The efficacy of botanicals including licorice in reducing side effects of cancer treatments like radiation and chemotherapy is, however, still little understood. Our goal was to provide clear information and emphasize the possibility of using drugs derived from licorice as part of a chemotherapy treatment plan for gastrointestinal inflammations. By improving QoL, this method has the potential to increase treatment adherence. The HPTLC finger printing and marker study lends credence to the idea that GG could improve patients' well-being by decreasing chemotherapy-induced side effects, especially those pertaining to the GI system. To begin evaluating the herb's potential, this study will serve as a beginning step.

KEYWORDS: Chemotherapy, GI inflammation, side effects, Prebiotics, Glycyrrhiza

INTRODUCTION

One of the main causes of mortality before one's time and a major obstacle to raising life expectancy in practically every nation on the planet is cancer. OneRadiation treatment, cytotoxic chemotherapy, and surgery have historically been the cornerstones of cancer treatment [1]. However, additional side effects from cancer treatment regimens, particularly from chemotherapy and radiation, include fatigue (50~90%), chronic pain (50~70%), oral mucositis, anorexia (~85%), gastrointestinal toxicity, hepatotoxicity, nephrotoxicity, insomnia (30~60%), oedema, depression/anxiety (24%/24%), or constipation (30~80%) [2–4].One of the main causes of cancer-related deaths is recurrence, which happens when tumours grow resistant to traditional chemotherapeutic medicines or even new targeted medications over time. Finding additional therapeutic agents is therefore imperative [5]. Furthermore, several natural products have the capacity to lessen side effects brought on by radiation and chemotherapy. Several symptoms, including nausea and vomiting, diarrhoea and constipation, and immunodeficiency, negatively affect a patient's quality of life (QoL) after receiving chemotherapy or radiation treatment. Chemotherapy's chemical components, which are mostly extracted from plants, are crucial for the treatment of cancer [6]. One of the main causes of cancer-related deaths is recurrence, which happens when tumours grow resistant to traditional chemotherapeutic medicines or even new targeted medications over time.

Mechanism of chemotherapeutic agents to cause GI inflammation

Chemotherapeutic drugs have a well-established mechanism and capacity to treat cancer cells. Early mucositis is characterized by the production of reactive oxygen species (ROS), and this starts right after chemotherapy is administered[7,8]. These in turn lead to the activation of nuclear factor-B (NF-B), a transcription factor associated with conditions that foster an inflammatory response. "Ulceration and inflammation," the fourth phase, is when the mucositis first manifests clinically [9]. This is the phase in which the permeability of the epithelial layer increases, permitting the translocation of bacteria into the bloodstream. Severe pain and discomfort are experienced along with the possibility of subsequent problems like septicemia and malnourishment. Other alterations that take place during the ulcerative stage include villus atrophy and crypt ablation, which decrease the amount of digesting enzymes in the gut and increase intestinal permeability. These changes also limit

the size of the intestine's overall absorptive surface. These adjustments then influence the gut microbiota [10].

Prebiotics is a relatively new subject being studied by researchers as a potential remedy for some drawbacks. Prebiotics are substrates that the host's microbes specifically use to provide health benefits [11]. They do not undergo metabolism in the human gut, but they do encourage the development and activity of good bacteria, which keeps the intestines of the body in balance[12]. Prebiotics, which include Lactobacillus and Bifidobacterium, suppress the growth of pathogenic bacteria, and increase the most common good bacteria for human health [13]. Prebiotics most used are lactose, oligofructose, inulin, and mangan-oligosaccharides.

These are not digestible carbohydrates;therefore the upper gastrointestinal tract does not metabolize them. Instead, they pass through the ileum and colon, where local microbes ferment them [14]. They also work by covering or stacking the receptors on the surface of the host. They produce bacteriocins, which help to kill dangerous germs. With the help of non-digestible carbohydrates, favourable bacteria generate short chain fatty acids, which provide energy to epithelial cells and help control immune response and metabolic processes. [15,16].

Liquorice as a choice of drug

Liquorice is a member of the genus Glycyrrhiza, and its dried roots and rhizomes are known as radix glycyrrhiza (RG). Commonly used as a natural sweetener, liquorice's triterpenoid saponins—particularly glycyrrhizic acid, which is one of the plant's main components and bioactive ingredients are employed in herbal medicine [17].

Radiation and chemotherapy, which cause mucosal irritation during clinical cancer treatment, quickly kill high proliferative cells, including cancer cells that are proliferating and dividing epithelial cells [18]. The most frequent adverse reaction to cancer treatment in people with head and neck cancer is mucositis. Glycyrrhiza aqueous extract was given to head and neck cancer patients on the first day of radiation therapy in a double-blind clinical experiment. It was discovered that this reduced the grade of mucositis and mucosal irritation following intervention [19]. Glycyrrhiza's anti-inflammatory properties may prevent macrophage activation, lower prostaglandin E2 levels, and reduce macrophages' release of free radicals [20]. Additionally, it can reduce reactive oxygen species and scavenge free radicals due to its antioxidant effect [21].

Reviewed MOA of Liquorice

During chemotherapy, GI Adverse Reaction Toxicology causes gastrointestinal side effects include nausea, vomiting, and diarrhoea [22]. A patient's level of discomfort may vary depending on the kind of chemotherapy, how long it takes, and how well they tolerate it. Among the emetogenic reagents are capecitabine [23], cisplatin, doxorubicin, and carboplatin [24]. PHY906 can inhibit the pathways that trigger intestinal inflammation caused by CPT-11, such as NFκB, cyclooxygenase-2 (COX2), and inducible nitric oxide synthase (iNOS) [25].

With reference to the above utility and applications of the drug it was selected as a study sample and initial phramacognostic studies were performed followed by the HPTLC finger print analysis and marker studies using a new solvent system.

Previous research has supported the role of Liquorice as potential herb for supporting the gut health and gastrointestinal flora [26]. To the best of our knowledge, there are no reports in the literature depicting effect of Glycyrrhiza on intestinal microflora from prebiotic aspect utilising the Lactobacillus species. In view of this, in the present study, effect of Glycyrrhiza on lactobacilli strain Lactobacillus acidophillus (LA), was evaluated by measuring their growth kinetics [27].

In lieu of this the active content of the extract used was being analysed by HPTLC finger print and marker study.

MATERIALS AND METHODS

For microbial study:

LA (ATCC 14931) were provided by the Department of microbiology, Dr. D. Y. Patil ACS College, Pune. Glycyrrhiza glabra aqueous extract was procured from the Amsar labs, Indore. The primary pharmacognostic parameter were analysed for the extract. All the media and chemicals were of analytical grade and purchased from HiMedia, Mumbai, India.

For HPTLC study:

A Camag HPTLC system equipped with an automatic TLC sampler (ATS4), TLC scanner 3, and integrated software winCATS version 1.2.3 was used for the analysis. HPTLC was performed on a precoated silica gel HPTLC 60F254 (20 cm X 20 cm) plate of 200 lm layer thickness for the quantification of glycyrrhizin and glycyrrhizinic acid in Glycyrrhiza glabra.

Method for Growth stimulating activity:

Microbial strains, media, and growth condition:

For the present study, microbial cultures of LA (ATCC 14931) were used. Lactobacilli strains were cultured in deMan-Rogosa Sharpe (MRS) broth and in reconstituted MRS broth and incubated at 37°C for 24 h in microaerophilic condition using candle jar. In candle jar method, a candle was placed in a desiccator containing plates such that the candle utilized the oxygen present in the desiccator to create a condition with low oxygen content. E. coli was cultured in MacConkey broth at 37°C for 24 h. For all the study protocols, an overnight (24 h) culture of each microbial strain was used [28].

Preparation of lactobacilli strains

For the preparation of LA culture, a single colony of lactobacilli previously-stored at −20°C on MRS agar plate was transferred to 30 ml of MRS broth and incubation was carried out at 37°C for 24 h in microaerophilic condition. The broth was centrifuged to obtain a pellet. The pellet was further re-suspended in MRS broth to obtain the turbidity of suspension adjusted to 0.5 McFarland standards. The inoculum concentration was approximately 1.5 × 110 cfu/ml as per inoculum density [29].

Sample for microbial study was added with Volume of inoculum – 1ml in 20ml MRS 24hr old culture done aseptically. Sample was made with three different doses named GG1 - 150mg/kg, GG 2- 300mg/kg, GG 3- 600mg/kg.It was divide in to different groupControl group different conc were made and added to reconstituted MRS without LA, Test group - different conc were made and added to reconstituted MRS with LA, Normal Control- Normal MRS media with LA, Experimental Control- Reconstituted MRS with LA [30].

Microbial strains, media, and growth condition. For the present study, microbial cultures of LA ATCC were used. Lactobacilli strains were cultured in de Man-Rogosa Sharpe (MRS) broth or in reconstituted MRS broth and incubated at 37°C for 24 h.

Preparation of lactobacilli strains:

A single colony of lactobacilli previously-stored at −20°C on MRS agar plate was transferred to 30 ml of MRS broth and incubation was carried out at 37°C for 18 h in microaerophilic condition. The broth was centrifuged to obtain a pellet. The pellet was further re-suspended in MRS broth to obtain the turbidity of suspension adjusted to 0.5 McFarland standards. The inoculum concentration was approximately 1.5 × 110 cfu/ml as per inoculum density [31].

Effect of Glycyrrhiza glabra on growth of lactobacilli.

The growth kinetic of lactobacilli was carried out in MRS broth and reconstituted MRS broth supplemented with GG. In MRS broth, the cultivation of LA was conducted with addition of 2% (v/v) of the inoculum in 250 ml of sterile MRS broth aseptically [32]. The flasks were

then incubated at 37°C for 48 h in anaerobic condition. Samples were collected after every 2 h to determine optical density at 600 nm. Each experiment was conducted in triplicate [33].

pH

The pH was measured by a calibrated electronic digital pH meter [34].

Method development for HPTLC

- The High-Performance Thin Layer Chromatography analysis was carried out on 20x10cm precoated silica gel aluminium plate 60F254 (E. MERCK, Germany). The sample extracts were applied to the plates as 6mm bands, under a stream of nitrogen, by means of a CAMAG (Switzerland) Linomat V semiautomatic sample applicator fitted with a 100µl HPTLC Hamilton syringe [35].
- Linear ascending development to a distance of 8cm was carried out in 20x10cm twin trough chamber saturated for 30mins at room temperature (25ºC±2) with 20ml mobile phase as Ethylacetate: Methanol: Water: Formic acid (9:1:1:0.5). The banding patterns were visualized in 254nm, 366nm and white light and Densitometric scanning was performed with Camag TLC scanner III in the reflectance absorbance mode at 540 nm after spraying with either 10% Sulphuric acid or Anisaldehyde Sulphuric acid and analysed by Win CATS software (1.3.0 Camag) [35,36].
- The developed chromatograms at 254nm were then compared based on the intensity of the spots obtained to finalize the best solvent for the extraction of the samples, best mobile phase for development of chromatograms and the best derivatization agent for visualizing spots.
- These results were then applied to prepare a final HPTLC fingerprint containing all the collected 24 root samples and invitro samples of *Glycyrrhiza glabra* were used. The final chromatogram was then observed to find the sample having maximum phytoconstituents by observing the spots. The plates were then scanned at 254nm and 366nm. The plates were derivatised by two different reagents where 1 was NP reagent and 2 was ASR reagent. Further the peaks were analysed to estimate the amount of Glycyrrizinin each of the samples [35, 37].
- The marker study was analysed using the standard ofGlycyrrizin procured from Yucca Chemicals.Sample applied qty 7.0 µL, Sample dissolved in water and methanol with mobile phase as GAA: Toluene: Ethyl acetate: Methanol: Formic acid (0.5:4:4.5:2:0.5)

Derivatisation of the plate was done by NP reagent and heat at 100 °C for 3 min, Reagent name ASR Reagent.

- **RESULTS AND DISCUSSION:**

Finger printing analysis of *Glycyrrhiza glabra*.

The derivatised plate showed different R_f at 0.16, 0.25, 0.41, 0.60 that confirms the presence of Glycyrrhizin (figure 1, figure 2) in the *Glycyrrhiza glabra*.extract and presence of Glycyrrhizinwas confirmed by comparing the R_f the marker GlycyrrhizinR_f 0.17 (figure3, figure 4).

Effect of GG on Growth of *Lactobacilli Acidophillus*

Effect of GG on growth of LA is shown in Figures 5. Addition of drug had significant ($P < 0.005$) effect on the growth of LA as well as compared to control. Maximum growth was observed during 2 to 28 hr (Table 1). A significant difference in growth of lactobacilli was observed with change in concentration of GG. An increase in GG concentration from dose 1 to 2 significantly increased the OD cultures which demonstrated the ability of GG to stimulate lactobacilli growth. Further increase in GG concentration (above dose 3) did not significantly increase OD as compared to dose 2 (Figure 5). Hence, concentrations of GG above dose2 were not considered in the study.

Effect on pH.

With increase in GG concentration, there was an increase in lactobacilli cell concentration resulting in increased production of lactic acid that is responsible for reduction in pH. After 24 h of incubation LC reduced the pH of media to 5.6 ± 0.07 and LF reduced to 5.4 ± 0.07 at Dose 3 concentration.

CONCLUSION

Various chemotherapeutic agents are known to disturb the gut health in cancer patient and eventually leads to secondary complication of muscle cachexia and fatigue that affects the quality of life [38]. The study was designed to investigate the prebiotic potential *of Glycyrrhiza glabra*on intestinal lactobacilli [39]. GG showed growth stimulative in vitro effect on LA that is indigenous strains of human Gut microflora where increase in

concentration showed a decrease in pH, increase in lactic acid production, which eventually improves the gut health [40]. On E.Coli the antimicrobial effects was seen and this contributed in improving the count of healthy microbes in gut. According to the HPTLC data the above study concludes presence of glycyrrhizin in the capable to show the desired effects [41]. Hence as per the above study the prebiotic potential of GG for prophylactic and therapeutic effect in gut health may be concluded where and extensive study on different strains of microbes remains as a thrust area.

AUTHORS CONTRIBUTION

All the authors contributed equally as Rashmi C Yadav holds and expertise in HPTLC analysis, Aarti Supekar holds expertise in microbials studies, and Dr.SS Bhujbal stands as guide for overall concept and execution of the same.

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CONFLICTS OF INTEREST None

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TABLES AND FIGURES

TABLE

Table 1: Effect of *Glycyrrhiza glabra*on growth of *Lactobacillus acidophillus*observed by optical density at 600nm at different time period.

Samples	**Control**	**Test**						
Time slot (hr)	0	0	2	4	6	8	24	48
C1	1.13	1.14	1.42	1.45	1.48	1.51	1.86	3.21
C2	1.17	1.19	1.38	1.43	1.32	1.39	1.58	2.96
C3	1.19	1.20	1.36	1.38	1.54	1.65	1.67	3.10

FIFURES

Figure 1: CCD image of TLC plates of *Glycyrrhiza glabra* Extract scanned at 254nm and derivatisation 1 with NP reagent and derivatisation 2 with ASR reagent was performed.

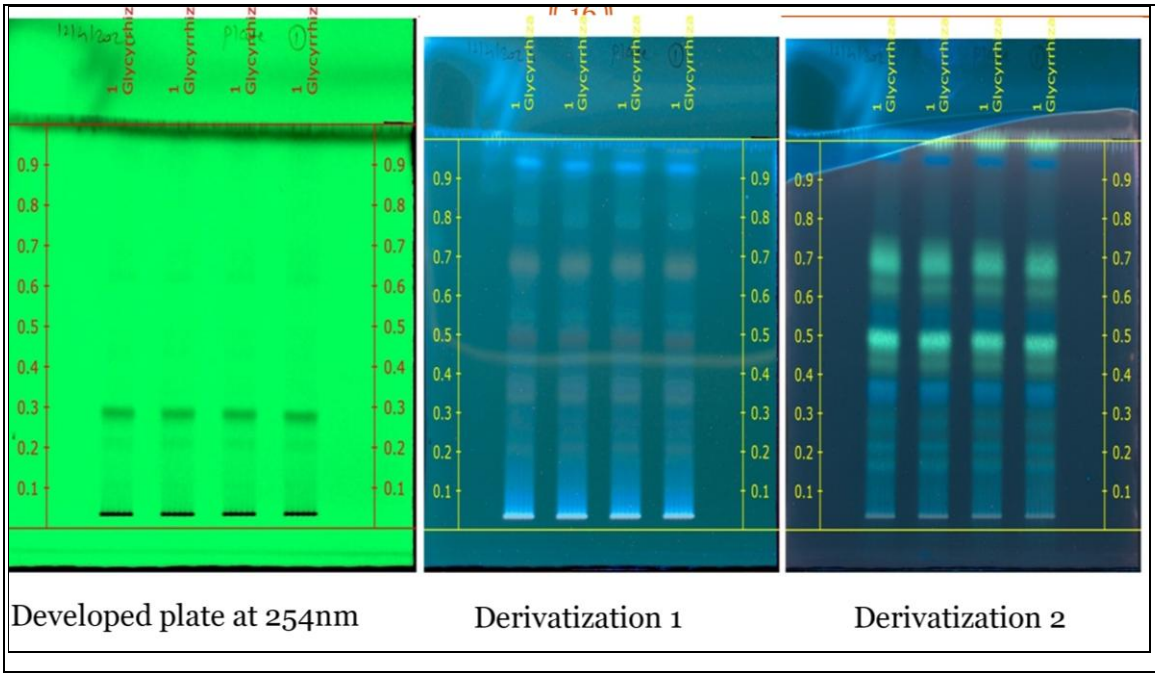
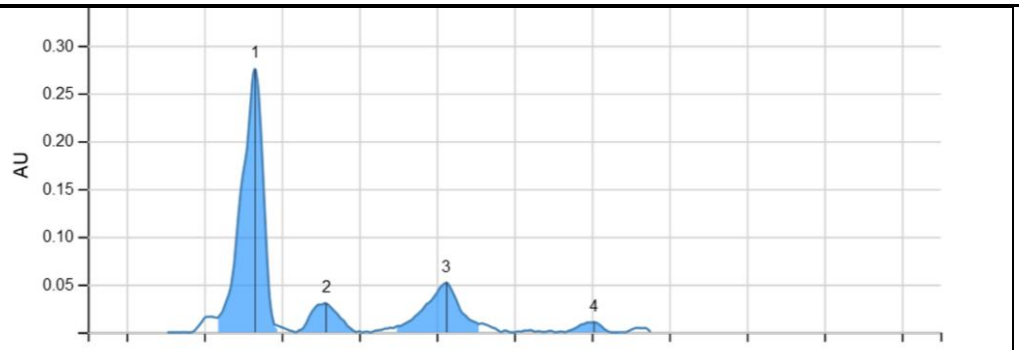
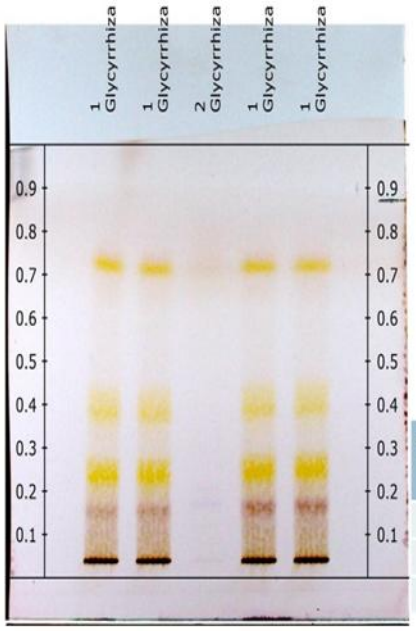


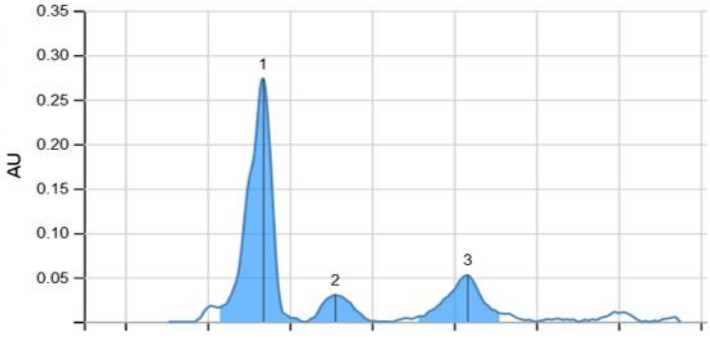
Figure 2: 2D graph and observation table showing the presence of *Glycyrrhizin* with peak numbered as 1,2,3,4 with specific R_f.



Peak #	Start		Max			End		Area	
	R_F	H	R_F	H	%	R_F	H	A	%
1	0.117	0.0153	0.165	0.2756	74.71	0.196	0.0035	0.00894	67.58
2	0.218	0.0000	0.257	0.0305	8.27	0.296	0.0003	0.00124	9.37
3	0.346	0.0053	0.412	0.0521	14.12	0.454	0.0086	0.00269	20.33
4	0.554	0.0000	0.603	0.0107	2.91	0.629	0.0000	0.00036	2.72

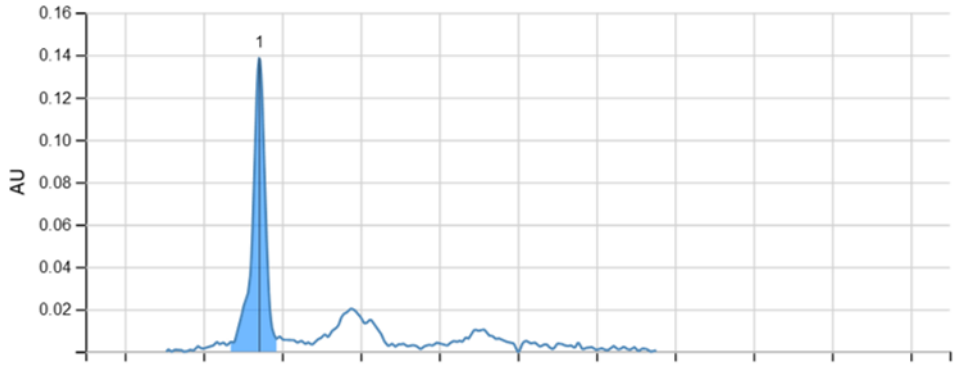
Figure 3: CCD image of TLC with *Glycyrrhiza glabra* labelled as track 1and, marker standard *Glycyrrhizin* labelled as track 2 extract plate. Scanned at 254nm and derivatised with ASR reagent.





Peak #	Start		Max			End		Area	
	R_F	H	R_F	H	%	R_F	H	A	%
1	0.114	0.0161	0.168	0.2736	76.74	0.218	0.0000	0.00913	69.78
2	0.218	0.0000	0.256	0.0304	8.52	0.300	0.0007	0.00128	9.81
3	0.356	0.0056	0.417	0.0526	14.74	0.461	0.0089	0.00267	20.42

Figure 4: 2D Graph and observation table showing presence of marker standard with *Glycyrrhizin* specific R_f.



Peak #	Start		Max			End		Area	
	R_F	H	R_F	H	%	R_F	H	A	%
1	0.131	0.0031	0.171	0.1386	100.00	0.193	0.0061	0.00267	100.00

Figure 5: 2D graph depicting the effects of different concentration of aqueous extract of *Glycyrrhiza glabra*on *Lactobacillus acidophillus*

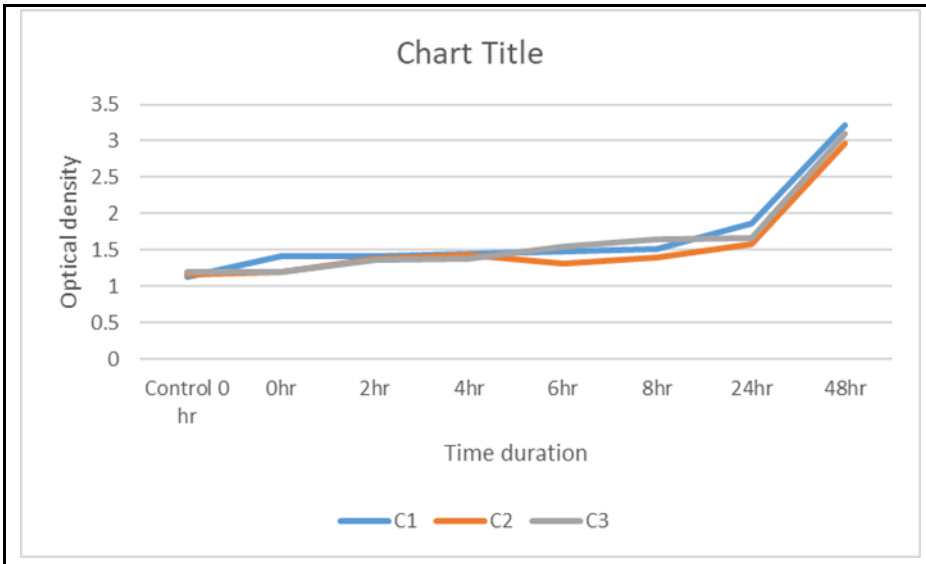


Chart Title
Optical density
3.5
3
2.5
2
1.5
1
0.5
0
Control 0 hr
0hr
2hr
4hr
6hr
8hr
24hr
48hr
Time duration
C1
C2
C3