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RESEARCH ARTICLE

Evaluation of Prebiotic Potential and HPTLC Analysis of *Withania somnifera* with Application in Improving the Chemotherapy-induced GI Side Effects

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Abstract

Background: Chemotherapy is a known choice of treatment for Cancer which has the aspect of producing strong adverse effects affecting the quality of life. To apar these various options are available wherein prebiotic use is emerging. An important herb Ashwagandha, derived from the dried mature roots of *Withania somnifera* (WS) Dunal, from the Solanaceae family (genus *Withania*), is utilised as a study specimen. **Aim:** The present study focuses on the determination of the prebiotic potential of the WS as a growth stimulant using *Lactobacillus acidophilus* (LA) with the support of HPTLC fingerprinting and marker study. **Method**: The growth analysis of WS was done using LA under microaerophilic conditions for 48hrs. The activity of WS is being confirmed using HPTLC m fingerprinting and marker study. **Results:** The extract showed the presence of the withaferin A with Rf of 0.364 using a solvent system (Methanol: Ethylacetate: Formic acid (1:9:0.5) and confirmed with standard marker using a solvents system of (Toluene: Ethyl acetate: Methanol: GAA (5:3.5:1:0.5). **Conclusion:** The growth of LA is stimulated showing an increase in lactic acid concentration and pH. HPTLC fingerprinting and marker study showed the presence of desired withanolides.

Keywords: Gut Microbiota, HPLTC, *Lactobacillus acidophilus*, Prebiotic, *Withania somnifera*

1. Introduction

Cancer is the leading cause of mortality on a global scale, accounting for nearly 10 million deaths in 2020, or almost one in every six deaths, according to the WHO[1]. The National Centre for Health Statistics projects that the United States will experience 1,958,310 new cancer cases and 609,820 cancer-related fatalities in 2023[2]. The causes of cancer are found to be variants. Nutrition affects 10% of fatal cancers, whereas tobacco use affects 22% of fatal cancers. 90-95 % of cancer cases are caused by environmental factors such as drug abuse, poor nutrition, stress, inactivity, and environmental pollutants[3,4]. The remaining 5–10 % of cancer cases are brought on by inherited cancer genetics.

Intensive chemotherapy is employed, comprising either a solitary medication or a combination thereof[5]. In certain instances, combination therapies are required to maximise treatment efficacy, notwithstanding the heightened likelihood of severe adverse effects that diminish the quality of life throughout and after chemotherapy[6,7].

While the potential side effects and risks of chemotherapeutic agents are duly acknowledged, conventional medications are consistently offered as a means of mitigating these effects. This will ultimately improve their quality of life, confidence, and faith, as well as streamline their daily activities[8]. Antiemetic, anti-inflammatory medications pose certain side effects like nausea, sleep disturbances, alterations in temperament, excessive drowsiness, hallucinations, and lethargy[9]. Antidiarrheals, including loperamide and diphenoxylate, are administered to treat diarrhoea. However, these medications have been linked to

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adverse effects, including nausea, vomiting, abdominal or stomach cramps, and at higher doses, excessive drowsiness, hallucinations, and lethargy. Though the above adverse effects are profound in their stages GI inflammation and muscle fatigue were found to be prominent during and post chemotherapy[10].

Chemotherapeutic agents' capacity and mechanism to eliminate cancer cells have been thoroughly demonstrated. In the early phases of mucositis, which initiates promptly following chemotherapy administration, Reactive Oxygen Species (ROS) are generated[11,12]. The activation of the transcription factor nuclear factor-B (NF-B) is a consequence of these, and it is associated with conditions that stimulate the inflammatory response. During the fourth phase, which is referred to as "ulceration and inflammation," mucositis manifests itself clinically for the first time[13]. Consequently, the intestinal microbiota experiences alterations due to these modifications[14].

Furthermore, an understanding of cryptal stem cells and progenitor cells reveals that apoptosis, one of the most significant issues with chemotherapy, inhibits cell mitosis and amplification[15]. Variables such as the cytostatic medication employed influence the extent of apoptosis and local cryptal modifications. The cytostatic doxorubicin (DOX), to give an illustration, is linked to the generation of reactive oxygen species and impairment of mitochondrial function[16].

Additionally, the epithelial villi are destroyed by the powerful metabolic response, which typifies these effects. The inflammation is further amplified as a result of this series of events. Some have postulated that variations in the expression of proteins that promote or inhibit cell death, such as Blc-2, which is thought to increase the rate of cell death in the microintestinal area, are to blame for the observed damage differences[17]. A prebiotic is a substrate that the host microbes use selectively to their advantage, leading to improved health[18]. These are not metabolised in the human gut, but they stimulate the growth and activity of beneficial bacteria, thereby balancing the organism's intestine[19]. Prebiotics consisting of the genus *Bifidobacterium* and *Lactobacillus*, which inhibit the proliferation of harmful bacteria, amplify the most used beneficial bacteria for human health[20]. Mannan-oligosaccharides, lactose, inulin, and oligofructose are the most frequently employed prebiotics.

The upper GI tract does not metabolise these indigestible carbs; instead, they make their way to the ileum and colon, where native microbes ferment them[21]. Another way they work is by covering the receptors on the host's surface in layers. They help destroy dangerous germs by producing bacteriocins. The energy source of epithelial cells, beneficial bacteria use non-digestible carbs to make short-chain fatty acids; these acids help regulate metabolic function and modulate immunological response[22,23].

The predominant *Lactobacilli* and *Bifidobacterium* species found in a healthy human gut are crucial for promoting gut health[24]. The creation of a biofilm on the intestinal epithelium, the release of organic acids, the generation of antibacterial compounds, and the stimulation of the host's immune system by Lactobacilli are all essential components of the defence mechanism and offer protection against pathogens[25]. Dysbiosis, a condition marked by a decline in the number of lactobacilli and an increase in the growth of pathogenic bacteria, is caused by any alteration in the local microflora[26]. Although there are well-established reports on prebiotics and probiotics used together to deal with these adverse effects majorly used orally to ensure the restoration of microbiota[27,28].

There are around 3000 species in the 84 genera that make up the Solanaceae family of plants. Traditional medicinal systems in South and Southeast Asia, such as Ayurveda and Unani, make heavy use of plants from the Physalis and *Withania* genera. *W. somnifera* L. Dunal (Solanaceae), more often referred to as "*Ashwagandha*," is a key component of Ayurvedic remedies for a variety of conditions, including inflammation, cancer, ulcers, infections, and senile dementia. It is also an antioxidant and anti-tumor[29,30].

An intact or rearranged ergostane framework, with C-22 and C-26 properly oxidised to create a six-membered lactone ring, is the building block of the withanolides, a class of naturally occurring C28-steroidal lactones[31].

Concerning the above utility and applications of the drug it was selected as a study sample and initial pharmacognostic studies were performed followed by the HPTLC fingerprint analysis and marker studies using a new solvent system[32].

Prior studies have provided evidence in favour of the potential of *Withania* as a herb to promote digestive

health and flora in the gastrointestinal tract[33]. Currently, no published reports detail the prebiotic impact of *Withania* on intestinal microflora using *Lactobacillus* species, Hence, the current investigation assessed the impact of *Withania* on the growth kinetics of the *Lactobacillus acidophillus* (LA) strain of bacteria[34,35].

Instead of this the active content of the extract was analysed by HPTLC fingerprint and marker study.

2. Materials and Methods

2.1 Materials

For microbial study: LA (ATCC 14931) were provided by the Department of Microbiology, Dr. DY Patil ACS College, Pune. *W. somnifera* aqueous extract was procured from the Amsar labs, Indore. The primary pharmacognostic parameters were analysed for the extract. All the media and chemicals were of analytical grade and purchased from HiMedia, Mumbai, India. For the HPTLC study: The solvents were used of HPLC grade and instrument of Camag Linomats HPTLC using Wincats software.

2.2 Methods

2.2.1 Method for Growth Stimulating Activity

2.2.1.1 Microbial Strains, Media, and Growth Condition

The present investigation made use of microbiological cultures of LA (ATCC 14931) as the source material. To cultivate lactobacilli strains, deMan-Rogosa Sharpe (MRS) broth and reconstituted MRS broth were used. The strains were then incubated at 37 degrees Celsius for 24 hours in a microaerophilic environment using a candle jar. The candle jar method involves placing a candle inside of a desiccator that has plates. This is done in such a way that the candle makes use of the oxygen that is available in the desiccator, thereby producing an environment that has a low oxygen concentration. At 37 degrees Celsius for twenty-four hours, *E. coli* was grown in MacConkey broth. An overnight culture of every microbial strain was used for all the experimental protocols. This culture lasted for twenty-four hours[35].

2.1.1.2 Preparation of Lactobacilli Strains

One colony of lactobacilli that had been stored at -20 degrees Celsius on an MRS agar plate was transferred to 30 millilitres of MRS broth to prepare a culture of lactobacilli. The culture was then incubated at 37 degrees Celsius for twenty-four hours in a microaerophilic environment. After centrifuging the soup, a pellet was obtained from the mixture. To acquire the turbidity of the suspension that was adjusted to 0.5 McFarland standards, the pellet was re-suspended in MRS broth for subsequent use. The inoculum volume and strength was approximately 1.5 × 110 colony-forming units per millilitre, as determined by the inoculum density[35,36].

Sample for the microbial study was added with Volume of inoculum – 1ml in 20ml MRS 24hr old culture done aseptically. The sample was made with three different doses named WS1 -250mg/kg, WS2- 500mg/kg, and WS 3- 1000mg/kg. It was divided into different groups Control 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, in Normal Control- Normal MRS media with LA, the Experimental Control- Reconstituted MRS with LA[37].

Media, microbial isolates, and growth conditions. In the current investigation, microbial cultures of LA (ATCC) were utilised. Strains of Lactobacilli were cultivated in de Man-Rogosa Sharpe (MRS) broth or reconstituted MRS broth for twenty-four hours at 37°C.

2.1.1.3 Preparation of Lactobacilli Strains

Under microaerophilic conditions, a solitary colony of lactobacilli that had been cultured at -20°C on an MRS agar plate was transferred to 30 ml of MRS broth and incubated at 37°C for 18 hours. By separating the bouillon by centrifugation, a pellet was formed. Following this, the particle was reconstituted in MRS broth to attain a turbidity of 0.5 McFarland standards. The estimation of the inoculum concentration was 1.5 × 110 cfu/ml, which was determined by the inoculum density[35,38].

2.1.1.4 Effect of W. somnifera on Growth of Lactobacilli

The growth kinetics of lactobacilli were assessed in MRS broth and reconstituted MRS broth supplemented with WS. To culture, LA in MRS broth, 2% (v/v) of the inoculum was added aseptically to 250 ml of sterile MRS broth. The containers were subsequently subjected to a 48-hour incubation period at 37°C under anaerobic conditions. A systematic collection of samples was conducted at two-hour intervals to determine the

optical density at a specific wavelength of 600 nm. The experimental protocols were executed in triplicate.[35,39].

2.2.1.5 pH

The pH was measured by a calibrated electronic digital pH meter.

2.3 Method Development for HPTLC

The High-Performance Thin Layer Chromatography examination was performed on a precoated silica gel aluminium plate 60F254 (E.MERCK, Germany). Under the influence of a stream of nitrogen, the sample extracts were applied to the plates in the form of 6mm bands. This was accomplished with the assistance of a CAMAG (Switzerland) Linomat V semiautomatic sample applicator that was equipped with a 100µl HPTLC Hamilton syringe. A twin trough chamber saturated with 20 millilitres of a mobile phase consisting of methanol, ethyl acetate, and formic acid at a ratio of 1:9:0.5 was used. A spray reagent 10% Sulfuric acid or Anisaldehyde Sulfuric acid is used followed by densitometric scanning with a Camag TLC scanner III in the reflectance absorbance mode at 540 nm.

Based on these findings, a final HPTLC fingerprint was produced using all samples of *Withania somnifera*. To assess the amount of Withaferin in each sample, the plates were scanned and the peaks were analysed. The marker study was analysed using the standard of Withaferin procured from Yucca Chemicals. The sample applied qty 7.0 µL, Sample was dissolved in water and methanol with mobile phase as Toulene: Ethyl acetate: Methanol: GAA (5:3.5:1:0.5). Derivatisation of the plate was done by NP reagent and heat at 100°C for 3 min, Reagent name ASR Reagent.

3. Results and Discussion

3.1 Finger Printing Analysis of *Withania somnifera*

The derivatised plate showed different R_f at 0.26, 0.37, 0.46, and 0.49 confirming the presence of Withanolides (Figure 1) in the *W. somnifera* extract and the presence of Withaferin was confirmed by comparing the R_f of the marker Withaferin R_f 0.36 (Figure 2).

3.2 Effect of WS on Growth of *Lactobacilli*

The effect of WS on the growth of LA is shown in (Figure 3). The addition of the drug had a 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 the growth of lactobacilli was observed with a change in the concentration of WS. An increase in WS concentration from dose 1 to 2 significantly increased the OD cultures which

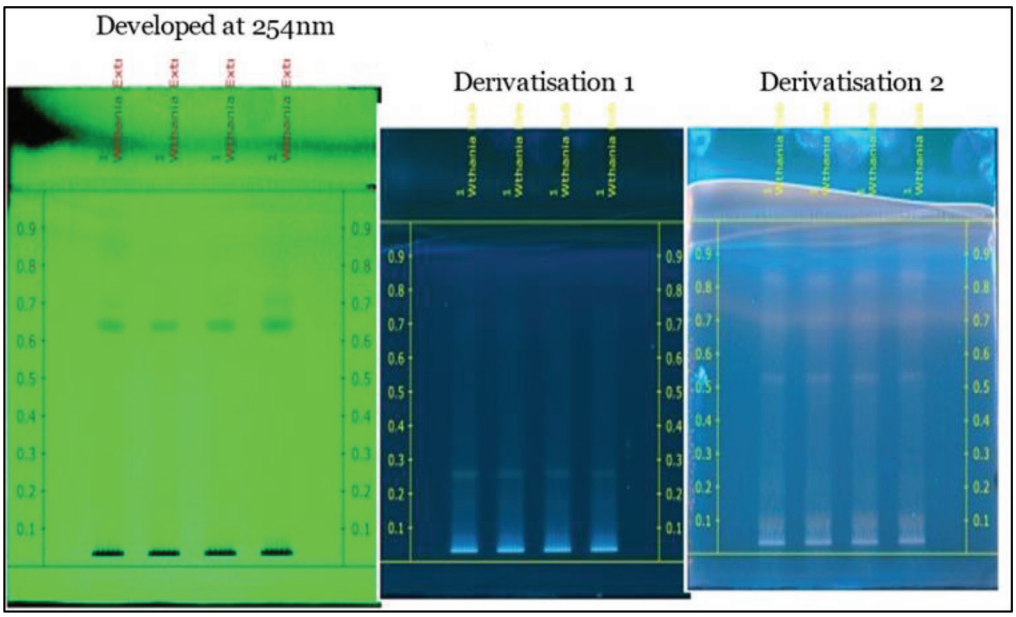

Figure 1. CCD image of TLC plates of *W. somnifera* extract scanned at 254nm and derivatisation 1 with NP reagent and derivatisation 2 with ASR reagent was performed.

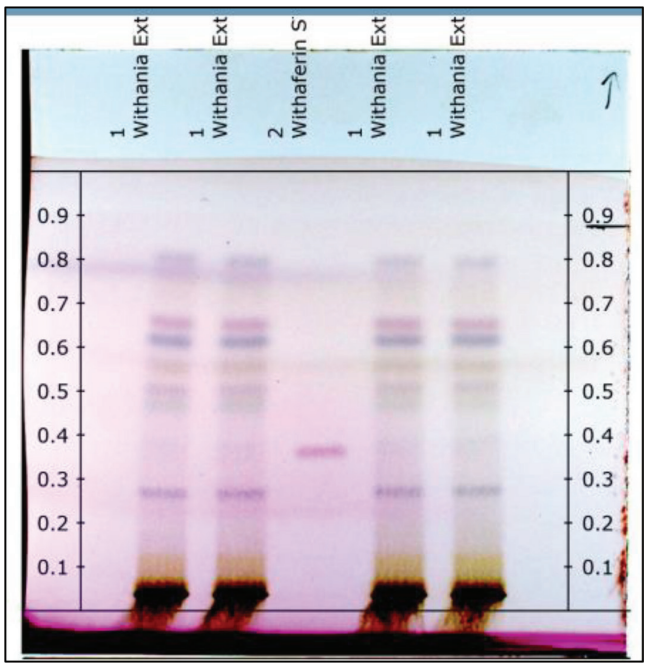

Figure 2. CCD image of TLC with *W. somnifera* labelled as track 1 and, marker standard Withaferin labelled as track 2 extract plate. Scanned at 254nm and derivatised with ASR reagent.

Table 1. Effect of *W. somnifera* on the growth of *L. acidophillus* observed by optical density at 600nm at different periods

Samples	Control	Test						
Time slot (hr)	0	0	2	4	6	8	24	48
C1	0.43	0.83	0.96	1.02	1.25	1.31	1.38	2.16
C2	0.57	1.35	1.35	1.69	1.97	1.33	1.35	2.26
C3	0.97	1.02	1.14	1.34	1.46	1.53	1.41	2.52

demonstrated the ability of WS to stimulate lactobacilli growth. Further, an increase in WS concentration (above dose 3) did not significantly increase OD as compared to dose 2 (Figure 3). Hence, concentrations of WS above dose 2 were not considered in the study.

3.3 Effect on pH

An elevation in WS concentration led to a concomitant increase in lactobacilli cell concentration, which subsequently stimulated the generation of lactic acid, the chemical compound accountable for the decrease in pH. Following 24 hours of incubation, the pH of

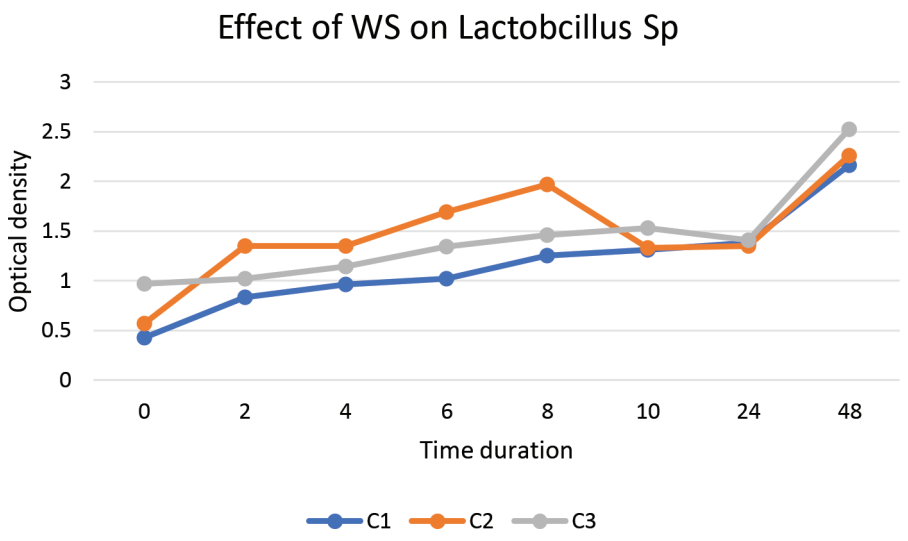

Figure 3. 2D graph depicting the effects of different concentrations of aqueous extract of *W. somnifera* on *L. acidophillus* (C1, C2, C3 indicates the different concentrations of extract).

the medium was decreased to 5.6±0.07 by LC and to 5.4±0.07 by LF at a dose of 3.

4. Conclusion

It is well recognised that several chemotherapeutic treatments can potentially disrupt the gastrointestinal health of cancer patients, which might ultimately result in secondary complications such as muscle cachexia and fatigue, which can hurt the quality of life[40]. The purpose of this study was to explore the prebiotic potential of *W. sominfera* on lactobacilli that are isolated from the intestinal tract[41]. WS demonstrated a growth stimulative *in vitro* effect on LA, which are indigenous strains of

human gut microflora. An increase in the concentration of WS resulted in a fall in pH and an increase in the production of lactic acid, which ultimately led to an improvement in gut health[42]. The antimicrobial effects were observed on *E. coli*, and this contributed to an increase in the number of beneficial microorganisms found in the gut. Based on the data obtained from HPTLC, the aforementioned study concludes that the presence of withaferin in the capable exhibits the effects that are intended. Consequently, according to the study that was shown earlier, it is possible to conclude that WS possesses the prebiotic potential to have both a preventative and therapeutic effect on gut health. However, there is still a need for more in-depth research on various strains of microbes[43,44].

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