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Dual impact of *Pulicaria undulata* extracts on colonic motility and *Blastocystis* structure: two factors linked to inflammatory bowel syndrome

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Abstract

Background Irritable bowel syndrome (IBS) is considered one of the most common diseases that affect the gastrointestinal tract. Several studies have shown that microbiota change and microorganisms like *Blastocystis* significantly contribute to IBS pathogenesis. *Blastocystis* resistance to commonly used antiparasitic drugs has emerged as an important subject for ongoing research. Introducing a new antiparasitic agent presents significant potential for improved management. *Pulicaria undulata (P. undulata*) extracts showed potential antiparasitic activity in previous research. Other reports clarified a possible gastrointestinal protective effect. However, no available studies have explored its potential activity against *Blastocystis* species (spp.) and its effect on colonic contractility.

Objectives This study was designed to investigate the impact of flower and leaf methanolic extracts of *P. undulata* on colon motility, which is directly related to IBS, and to assess their in vitro efficacy against *Blastocystis* subtypes isolated from symptomatic patients.

Materials and methods *P. undulata was* extracted and metabolites were analyzed. Isolated subtypes of *Blastocystis* spp. from collected samples were identified. Extracts effect on colonic motility and contractility was assessed in vitro using isolated colonic segments. In addition, cultured *Blastocystis* spp. was challenged with different concentrations of flower and leaf extracts as well as nitazoxanide. Structural, morphological, and ultrastructural alterations were evaluated using light and scanning electron microscopes.

Results The amplitude of colonic contractility decreased in response to *P. undulata* methanolic extracts, reflecting its possible spasmolytic effect on colonic motility, which was more noticeable with leaf than flower methanolic extracts. In vitro effect on ultrastructure of *Blastocystis* was seen with an obvious impact of both extracts on the shape and size of the parasite, which was mostly time and dose dependent. As for the molecular study, the most commonly isolated *Blastocystis* subtype was subtype 1. GC–MS analysis of silylated *P. undulata* flower and leaf methanolic extracts identified 46 metabolites. Flowers' methanolic extract exhibited greatly higher flavonoid concentration than leaves.

Conclusion The findings of this study emphasize probable therapeutic effects of *P. undulata* plant methanolic extracts in improving IBS symptoms by decreasing colonic motility while opening avenues for future research to consider *P. undulata* as a safe natural treatment of the implicated co-infection of *Blastocystis*.

Keywords *Blastocystis*, *Pulicaria undulata*, IBS, Colon motility, Ultrastructure

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Introduction

Irritable bowel syndrome (IBS) is lately being recognized as a dysfunction of the gut–brain axis [1], resulting in chronic functional illness with symptoms like abdominal pain, discomfort, stool consistency, or motion frequency change. It typically coexists with other functional gastrointestinal diseases (FGIDs) and can affect quality of life, work efficiency, and represent an economic problem. The most recent Rome IV criteria classify IBS into four subtypes according to predominant symptom: constipation-predominant (IBS-C), diarrhea-predominant (IBS-D), mixed stool pattern (IBS-M), and undefined or amorphous subtype (IBS-U). The global prevalence of IBS is approximately 15.0% but varies by nation or population, with the highest (18.9%) in South America and the lowest (11%) in Southeast Asia. The occurrence in females (15%) is higher than in males (11%), which might be related to immunological malfunction or the capacity of female estrogen to raise intestinal sensitivity [2, 3].

It is considered a chronic non-organic disease characterized by disrupted colonic motility and increased blood levels of neuroendocrine signals such as calcitonin gene-related peptides. The predominant IBS-diarrhea subtype is associated with colonic hypermotility, which is often relieved by cannabinoids [4–6].

Although the exact pathophysiology of IBS is still unknown, it is thought to be connected to various factors as psychosocial status, food intolerance, infection, and vitamin D insufficiency. After COVID-19 pandemic, there were reports of the development of IBS as a part of Post-COVID-19 syndrome (PCS) [2, 3]. Thus, post-infection changes with associated chronic gut inflammation, microbiota changes, motility issues are expected to have a role in IBS progress. Environmental factors like air pollution are also thought to play a role. More commonly IBS is thought to be triggered by psychological factors such as stress and gut–brain interactions. It is observed among adult women and those under 50. Besides, hereditary factors such as genetics, immune factors, and related cultural habits and diet are currently suspected [7, 8]. A family history of IBS and experiences of childhood trauma—whether physical or sexual—are notably related to developing IBS, indicating that a gene–psychological environment interaction during childhood may play a role [1].

For diagnosis, patient history, including psychological, family, and dietary intolerance histories, as well as presenting symptoms like abdominal bloating, pain, constipation, or diarrhea are crucial for suspicion. Signs of other abdominal problems such as masses, intestinal obstruction, or local pain should be excluded. Additionally, digital rectal examination is helpful to exclude pelvic floor dysfunction. Laboratory investigations should include complete blood picture to exclude anemia, tests for inflammatory bowel disease (IBD) like C-reactive protein to detect inflammation, and tissue transglutaminase IgA to detect celiac disease. Endoscopy is recommended to exclude colon cancer in patients above 45 years with recent bowel function alteration, weight loss or rectal bleeding, and anemia. The first line of treatment for IBS is fiber and osmotic laxatives for constipation (ispaghula husks are preferred to bran fibers), loperamide for diarrhea, and antispasmodics for cramping or abdominal pain. Other drugs used to treat diarrhea include serotonin type 3 (5-HT3) antagonists, effective in cases of functional diarrhea and IBS-D (especially for females not improving with 6 months of conventional therapy). As for constipation, osmotic laxatives, ileal bile acid transporter inhibition, chloride secretagogues, prokinetic agents, and ion channel blockers were used [9].

Psychotherapy proved beneficial, including cognitive behavioral therapy (CBT), relaxation therapy, multicomponent psychological therapy, hypnotherapy, and dynamic psychotherapy. Psychopharmacological treatment with a neuromodulator, such as a low-dose tricyclic drug, may be administered if there are other somatic comorbidities or psychiatric symptoms [10]. Alternative medicine (such as acupuncture and herbal products) and complementary non-pharmacological therapy like dietary exclusions, microbial modification of the gut have been tried. Exclusion diets, like sugar exclusion FODMAP (fructans, oligosaccharides, disaccharides, monosaccharides, and polyols), or adding prebiotics and probiotics, have been approached, as well as fecal microbiota transplant [11].

Blastocystis is a widely dispersed gastrointestinal protist, often reported in tropical and subtropical countries. This protist has a prevalence of up to 30–60% in developing countries [12]. Although many carriers are asymptomatic, some individuals report experiencing stomach pain, vomiting, lack of appetite, nausea, flatulence, rash on the trunk or extremities, and decreased weight during the pubertal period. *Blastocystis* is commonly isolated from patients with gastrointestinal diseases, such as gastroduodenitis, gastric cancer, and, much more frequently, IBS. The relationship between IBS and *Blastocystis* has attracted wide attention in recent years [13, 14].

The ability of *Blastocystis* to cause pathological changes remains debatable, and that is why, the decision to give treatment is doubtful. Nevertheless, it is generally accepted that treatment for *Blastocystis* is advised in cases presenting with severe symptoms, where fecal analysis indicates a high presence of *Blastocystis* cysts and no other apparent cause for the symptoms is identified [15]. Although metronidazole is the routine treatment for *Blastocystis* infections, multiple reports of therapeutic

failure have been documented, suggesting that some isolates can show drug resistance [16]. Nitazoxanide (NTZ) is one of the alternative therapies that have been used lately [17]. This encouraged the researchers to search for alternative therapies for *Blastocystis* infection, specifically studying other active natural agents.

Pulicaria undulata (P. undulata) is classified as a sunflower plant that is characterized by having an aromatic nature [18]. It is a member of the Asteraceae family, which includes over 80 species widely spread throughout Europe, Asia, and Africa [19]. As traditional remedies, plants in the genus *Pulicaria* have been used to cure a variety of ailments, including diarrhea, dysentery, intestinal diseases, menstrual cramps, inflammation, and back pain [20, 21]. Using Qualitative Ultra- Performance Liquid Chromatography with the ionization and mass analysis of Electrospray Ionization and Quadrupole-Time of Flight (UPLC-ESIQ-TOF) analysis, researchers identified 27 compounds classified as fatty acids, triterpenes, flavonoids, coumarins, and phenolics. In numerous studies, *Pulicaria* spp. have shown a noticeable impact on ulcers, microbes and fungi, as well as their antioxidant, and anticancer bioactivities. Another study revealed that *P. undulata* could guard rats against stomach ulcers caused by ethanol [22].

The current study aimed to assess the effects of *P. undulata* on the colonic motility of experimental mice and to assess the in vitro effect of *P. undulata* on the ultrastructure of *Blastocystis* in comparison with NTZ, the often-used treatment for *Blastocystis* infection.

Materials and methods

Therapeutics preparation

i. *Preparation of plant extracts: Pulicaria undulata* (L.) C.A.Mey was collected from Saudia Arabia, during August and September 2023. Plant identity was kindly authenticated by Therese Labib, senior botanist at El – Orman Botanical Garden. Dried flowers and leaves [10 g] were separately extracted with methanol [weight per volume (w/v)] in five successive portions [100mL each]. The resulting extracts were concentrated to dryness under reduced pressure at 40 °C using a rotary evaporator. The dried extract yielded 1.5 g, corresponding to a 15% w/w yield, and was subsequently used for biological activity assays and Gas Chromatography-Mass Spectrometry (GC/MS) analysis following Silylation [23].

ii. *NTZ preparation:* NTZ oral suspension [Nanazoxid 100 mg/5 ml] (Utopia Pharmaceuticals) was used to create a stock solution. Five milliliters were added to 5 mL of distilled water. One milliliter of the previous solution was dissolved in 9 mL of distilled water. For usage, 1 mL of this final stock had a concentration of 1000 µg (µg)/mL [24].

Blastocystis preparation

i. *Collection of stool samples:* Fresh fecal samples were collected from patients with symptoms who were experiencing different conditions like diarrhea, abdominal pain, and bloating, attending the Diagnostic and Research Unit of Parasitology, Faculty of Medicine, Cairo University. Eligible patients were 18–65 years old, could provide a fresh stool sample, and had not taken systemic antibiotics or oral antiparasitics in the past 8 weeks, nor probiotics, bismuth, or laxatives in the past 2 weeks. Females were not pregnant or breastfeeding. Samples that revealed co-infection with other parasitic species or gross blood were excluded. Also, patients reporting hematochezia within the past 12 weeks or diagnosed with enteric pathogens in the past 6 months (e.g., *Salmonella, Shigella, Campylobacter, Giardia, Cryptosporidium, Norovirus*) were not included. No samples were collected from patients who received systemic antibiotics or antiparasitic drugs within the past 8 weeks or form immunosuppressed patients (e.g., active cancer chemotherapy, organ transplant recipients, HIV).

ii. *Processing of Stool samples:* Stool samples were tested by a light microscope directly after collection, using both wet smear preparation in saline and staining it with Lugol's iodine solution, before and after adding formalin/ethyl acetate concentration to identify *Blastocystis* and eliminate and exclude the presence of other parasites in the sample. For *Blastocystis*-positive stool samples, a piece was cultured for in vitro study immediately that same day, while another was stored at −20 °C to be subjected to molecular genotyping.

iii. *In vitro culture of Blastocystis:* Culturing of positive *Blastocystis* stool samples was done in a test tube containing 5 mL of Jones' medium, 100 international unit (IU)/mL penicillin, 100 µg/mL streptomycin, and adding 10% horse serum [25]. Each cultured tube was incubated at 37 °C and checked daily for up to 72 h by light microscopy. The culture that fails to detect *Blastocystis* after 72 h was considered negative. Cultures were accepted for drug evaluation when *Blastocystis* numbers exceeded 1×10^3 vacuolar forms per mL [26].

Amplification and genotyping of *Blastocystis* isolates using nested polymerase chain reaction (PCR)

For deoxyribonucleic acid (DNA) extraction, 200 mg of each stool sample were processed using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) concurring with the manufacturer's instructions. After extracting DNA from stool samples, the small-subunit ribosomal RNA gene (SSU rDNA) was amplified using nested PCR. Consequently, two sets of primers were included in this study. The first primer set for the first reaction was: forward primer: A (5′-GCTTATCTGGTTGAT CCTGCCAGTAGT-3′) and reverse A (5′-TGATCC TTCCGCAGGTTCACCTA-3′). The second set of primers for the second reaction was: forward primer: B (5′-GGAGGTAGTGACAATAAATC-3′) and reverse primer: B (5′- ACTAGGAATTCCTCGTTCATG-3′). The PCR cycling conditions were: 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 54 °C for 1 min, and then 72° for 1.5 min. This is followed by a final extension cycle of 72 °C for 10 min. In the case of primer set B, the primer annealing temperature used was 50 °C instead of 54 °C. The PCR products [1100 base pairs (bp)] were separated on 1.5% agarose gel and visualized under ultraviolet (UV) transillumination [27].

Finally, a reproduced method of *Blastocystis* spp. subtype (ST) identification was achieved, by standardized subtype-specific Sequence-Tagged-Site (STS) primers for the more commonly suspected subtypes in local studies, as follows: **primer SB83** for subtype 1 (ST 1) with forward primer (5′-GAAGGA CTCTCTGACGATGA-3′) and reverse primer (5′-GTCCAAATGAAAGGCAGC-3′) and product size 351 bp; **primer SB227** for subtype 3 (ST 3) with forward primer (5′-TAGGATTTGGTGTTTGGAGA-3′) and reverse primer (5'-TTAGAAGTGAAGGAGATG GAAG-3') and product size 526 bp; and finally **primer SB336** for subtype 6 (ST 6) with forward primer (5′-GTGGGTAGAGGAAGGAAAACA-3′) and reverse primer (5′-AGAACAAGTCGATGAAGTGAGAT-3′) and product size 317 bp [28]. After staining with ethidium bromide, PCR products were separated on a 1.5% agarose gel electrophoresis and the amplified products were visualized using gel documentation system.

Therapeutics trials on IBS-related factors

This therapeutics study was done to detect the effect of different plant extracts versus NTZ on cultured *Blastocystis* spp. and to evaluate their impact on the motility of the intestine. The study was conducted in vitro in two phases.

In vitro experimental study of the extracts effect on colonic motility

In this phase, animal experimental protocol included 10 Wistar albino rats weighing 200–220 g per rat, obtained from the Animal House, Faculty of Medicine, Cairo University. Rats were acclimated for one week. Feeding, handling, and animal care were performed according to the guidelines of the ethical committees of Cairo University [Institutional Animal Care and Use Committee (IACUC) number **CU-III- F- 55- 24].**

To assess the effect of *P. undulata* extracts on colonic motility, 10 rats were sacrificed by cervical dislocation, and the whole colon was removed from each one of them. Then, it was cleaned from fat and mesentery and immediately immersed in warm, carbogenated Krebs solution (in mM:118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 NaH_2 PO_4, 1.2 $MgCl_2$, 11D-glucose, and 25 $NaHCO_3$). The solution was continuously gassed with O_2, and the pH ranged from 7.3 to 7.4 [29]. A segment (3 cm long) of the proximal colon (1 cm from the cecum) was removed from each isolated colon of each rat to have 10 colonic segments maintained living inside 50 ml of carbogenated Krebs solution continuously gassed with O_2 to be used for the production of a demonstration curve for colonic motility. Each isolated colonic segment was suspended in a 10 mL organ bath containing oxygenated Krebs solution at 37 °C to be assessed independently. The distal end of the colonic segment was tied around the mouth of a J-tube, and the ligated proximal end was secured with a silk thread to an isometric force displacement transducer. The preparations were equilibrated for 30 min after an established initial resting tension of 0.5 g.

Isometric developed tension was measured by a force transducer coupled with data acquisition system and analysis software (PowerLab, AD Instruments) using Radnoti single tissue organ bath 4/30, ML221 (Australia), force transducer (TR 1201 Pan lab, Spain) (Fig. 1).

The motility of each isolated colonic segment was detected as longitudinal muscle contraction (isometric tension) and recorded. First, tissue viability and contractility were tested by methacholine in a selected average dose of 50 µg/10 ml organ bath fluid [30]. Colonic motility recording continued afterward while testing the effect of DMSO, the vehicle used for extract preparation, at a dose of 0.1 ml. The leaf and flower extracts were tested at a selected average dose of 100 µg prepared by adding 0.1 ml (equivalent to 10 mg) of the dried extract to 1 ml DMSO and then diluting the mixture in 9 ml distal water. Only 0.1 ml of the acquired dilute was applied during testing in the 10 ml organ path fluid [31, 32]. NTZ was applied in a dose of 100 µg/10 ml organ bath fluid [33]. Sequence of motility recording was as follows: methacholine, vehicle, both extracts, methacholine after washing

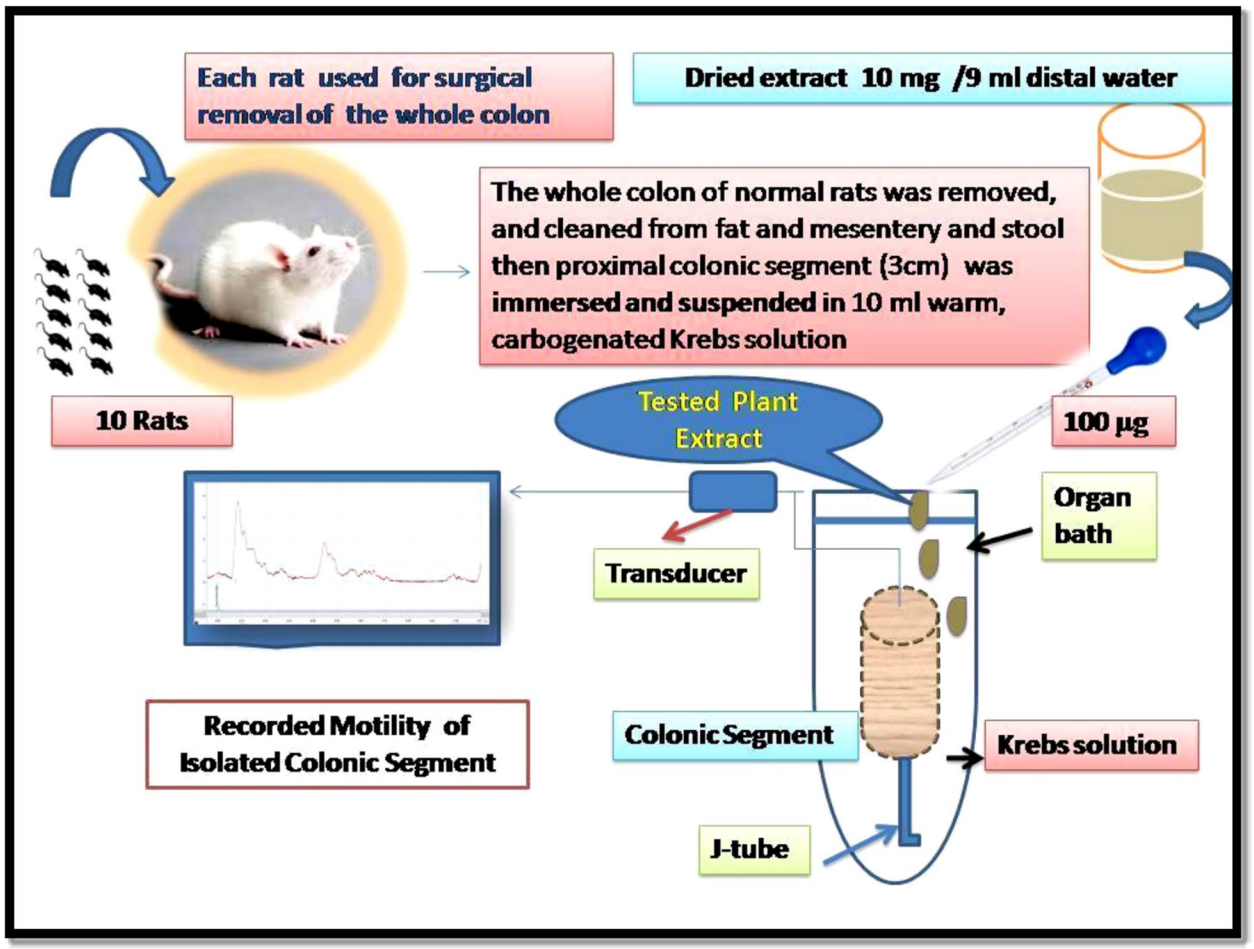


Fig. 1 A flow chart for the in vitro experiment to test the effect of *P. undulata* extracts on normal colonic motility

extracts, NTZ, and finally methacholine after washing NTZ. Methacholine and other agents were added to the bath in volumes less than 1% of the total bath volume.

In vitro study of the extracts effect on Blastocystis culture

i. Experimental design and processing

Culture tubes containing Jones medium that exhibited growth of *Blastocystis* were exposed to various concentrations of flower and leaf extracts of *P. undulata* and NTZ to evaluate their influence on the growth and ultrastructure of the parasite. Control cultures of the parasites that were not treated and negative control (culture media only) were subjected to the same technique used for the other tested cultures. Three culture tubes were prepared for every concentration of each; flower extract, leaf extract of *P. undulata,* NTZ, and non-treated cultures. The tested cultures were incubated at 37 °C to be examined by microscope after 24 h and 72 h of incubation. Parasites were exposed to a graded concentration of flower extract of *P. undulata* (100 and 400 µg/mL), leaf extract of *P. undulata* (100 and 400 µg/mL) [34–36], and NTZ 100 µg/mL [37] (Table 1).

ii. Assessment of the in vitro anti-*Blastocystis* activity of plant extracts and NTZ:

Table 1 Culture tubes and corresponding therapeutics concentration

Prepared culture tubes	Concentration of therapeutics
Control negative	Culture media only
Control positive	Untreated *Blastocystis* culture
NTZ	*Blastocystis* culture with NTZ 100 µg/mL
Leaf 100	*Blastocystis* culture with leaf extract 100 µg/mL
Leaf 400	*Blastocystis* culture with leaf extract 400 µg/mL
Flower 100	*Blastocystis* culture with flower extract 100 µg/mL
Flower 400	*Blastocystis* culture with flower extract 400 µg/mL

a **Light microscopy for the in vitro evaluation:** The effectiveness of the tested drugs on *Blastocystis* was assessed by spotting the changes in the features of cultured parasites using a light microscope.

b **Scanning electron microscope (SEM) for the in vitro effect on Parasite:** For the recognition of ultrastructural changes, 0.5 mL of sediment was used from each examined culture. All samples underwent the same processing steps (untreated control and cultures treated with variable concentrations of plant extract and NTZ). The sediment was first fixed in 3% glutaraldehyde for 2 h, followed by two washings with 0.1 molar (M) sodium cacodylate buffer (pH 7.2). A serial ethanol dehydration was done: 10, 20, 40, 60, 70, 80, 95, 100%, for 25 min each. The last step was repeated (100%), and then, the specimen was dried using a critical point dryer and mounted on a metal stub, which increases conductivity. Finally, coating the sample with gold was applied using a sputter coater and specimens were examined using a JEOL JSM 5200 SEM [38, 39].

Plant extracts biochemical analysis

i. **GC-MS profiling of silylated primary metabolites in *P. undulata* flowers and leaves extracts**

Methanolic extracts [100 µL] obtained from the flowers and leaves of *P. undulata* were dried completely under a nitrogen stream. The resulting residues were derivatized with 150 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA; Sigma, St. Louis, MO, USA) and incubated at 60 °C for 45 min, following the procedure outlined in a previous study [40]. Silylated derivatives were subjected to analysis using a Shimadzu GC-MS-QP 2010 system (Tokyo, Japan) equipped with a quadrupole mass spectrometer (Thermo-Finnigan SSQ7000). Chromatographic separation was achieved on an Rtx-5MS fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Restek, Bellefonte, PA, USA). Before injection, samples were diluted with n-hexane [1% volume per volume (v/v)] and introduced in split mode with a 1:15 split ratio. Each sample was analyzed in triplicate, and the mean peak areas were calculated. A homologous series of n-alkanes (C8–C18; Aldrich Chemical Company, USA) was injected under identical conditions to determine retention indices.

ii. GC–MS data processing:

The GC–MS data were processed using AMDIS software (www.amdis.net) to aid in peak deconvolution before metabolite mass spectra were matched to the NIST database. Peak identification was based on calculated KI using alkane standard C8-C40, spectral matching, and standards whenever available, following the exact protocol detailed in a previous study [41].

iii. Determination of total flavonoids:

The total flavonoid (TF) content was determined using a method adapted from a prior study [42]. A calibration curve was constructed using quercetin standards in the concentration range of 10–120 µg/mL. To determine the quercetin equivalent content in the extracts, the absorbance of each sample was recorded. For color development, 500 µL of either quercetin standard, sample extract, or methanol (as a blank) was mixed with 250 µL of aluminum chloride solution, followed by the addition of 250 µL of 1 M hydrochloric acid. After a 10-minute incubation period, the absorbance of the resulting mixtures was measured at 420 nm.

Statistical methods

Results of colonic contractility response were presented as mean ± SD. Parametric measurements were statistically analyzed using the Student t-test. Statistical significance was considered as $p < 0.05$. Microsoft office 10 for windows 8 was used to conduct the analysis.

Results

Effect of extract on the motility of the isolated colonic segments

Pulicaria undulata extract showed a significant inhibitory effect on the motility of the isolated colonic segments with either flower or leaf extract (Fig. 2). This inhibitory effect was more prominent with the application of leaf extract to the colonic segment. In addition, this effect has attenuated the normal response of the isolated colonic segments to methacholine. This attenuation was significantly more prominent in comparison with NTZ (Table 2).

These results can reflect the possible spasmolytic effect of both flower and leaf extracts on the motility of the colon.

Genotyping of *Blastocystis* isolates using nested PCR

The products of the nested PCR targeting the *SSU rDNA* gene of *Blastocystis* spp. were separated at 1100 bp on 1.5% agarose gel electrophoresis. Subsequently, the identification of *Blastocystis* spp. was achieved by standardized subtype-specific STS primers. Following staining with ethidium bromide, bands separated at 351 bp by 1.5% agarose gel electrophoresis, confirming *Blastocystis* ST1 (SB83) in all studied samples (Fig. 3).

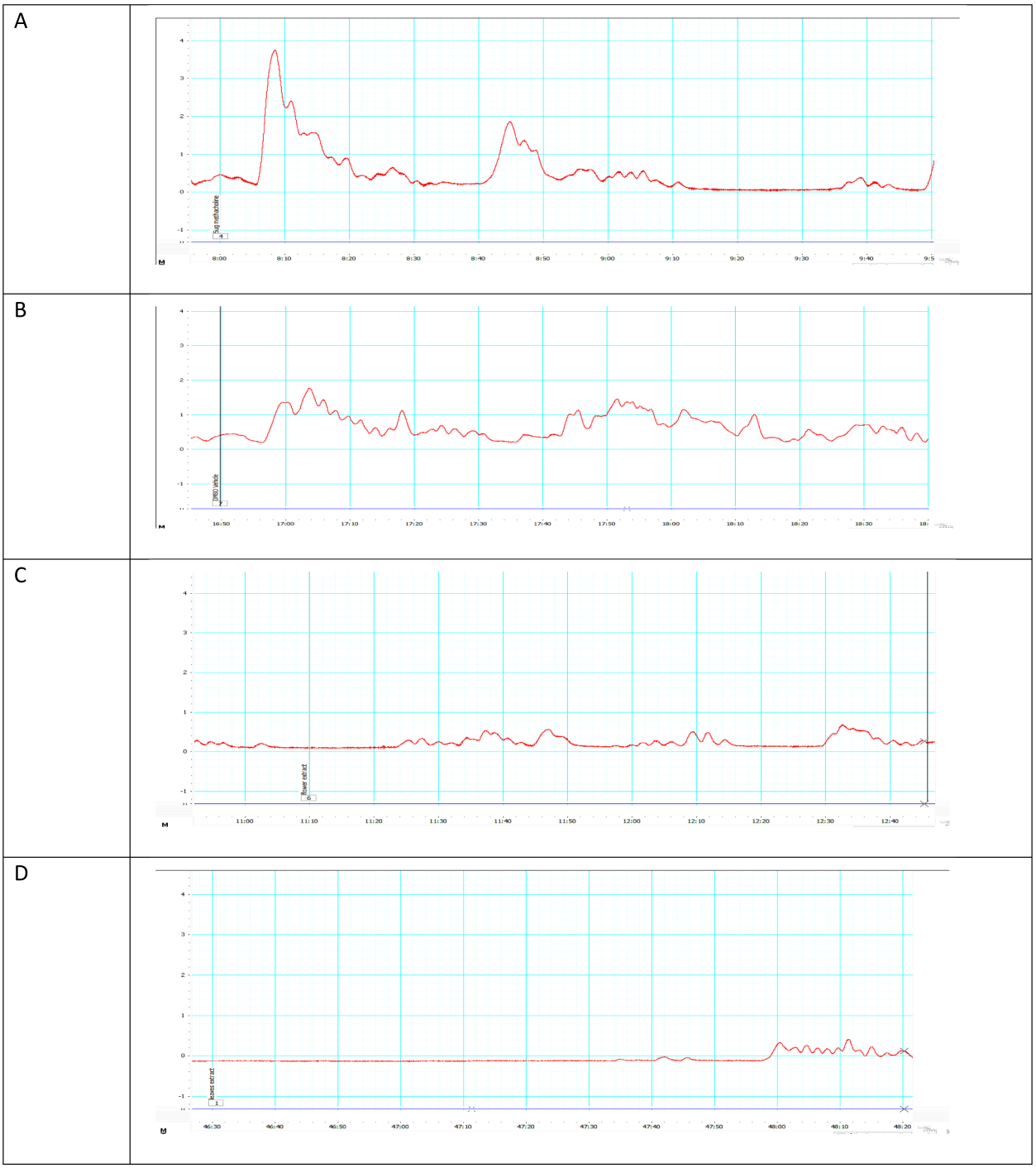


Fig. 2 Contractility response of colonic segment to investigated agents [A-G] showing: **A** normal response of colonic segment to 50 µg methacholine; **B** response of colonic muscle to adding 0.1 ml of DMSO to colonic strip, **C** colonic contractility after adding 100 µg of flower extract (it revealed inhibitory effect on colonic contractility, even after wash), **D** effect of adding 100 µg of leaves extract (exhibited decrease colonic contractility, even after wash); **E** represents addition of 50 µg methacholine again after washing leaf and flower extracts [showed decrease smooth muscle developed tension compare to previously recorded response in (**A**)]; **F** represents response of colonic segment to 100 µg of NTZ [restored intestinal contractility but to a lesser degree compared to (A)]; and **G** showed response of colonic segment for 50 µg methacholine, after washing NTZ, [increased contractility compare to (**E**), however, it didn't reach normal colonic motility recorded in (**A**)].



Fig. 2 continued

Table 2 Results of contractility response of colonic segment (colonic motility) to different investigated vehicle, drugs, and plant extracts:

Amplitude of muscle response per g (mean ± SD)						
Methacholine (Normal)	**DMSO**	**Flower extract**	**Leaf extract**	**Methacholine after plant extract**	**NTZ**	**Methacholine after NTZ**
4 ± 0.13	1.8 ± 0.12$^{\$}$	0.2 ± 0.08$^{\$}$	0.14 ± 0.02$^{\$}$	0.4 ± 0.05$^{\$\#}$	1.4 ± 0.17$^{\$}$	1.7 ± 0.14$^{\$}$

$ Significant difference compared to **normal methacholine response.**

Significant difference compared to the **methacholine response after NTZ.**

Effect of methanolic extracts on *Blastocystis* using light microscope (LM)

The major form detected during this study was the vacuolar form with an outer rim of cytoplasm containing nuclei. After adding plant extracts with different concentrations, a range of various changes and parasite sizes could be spotted with each; however, certain changes prevailed.

Leaf extract 100 µg/mL concentration

After exposure for 24 h, *Blastocystis* showed some irregularity in outline and a few showed singular blunt

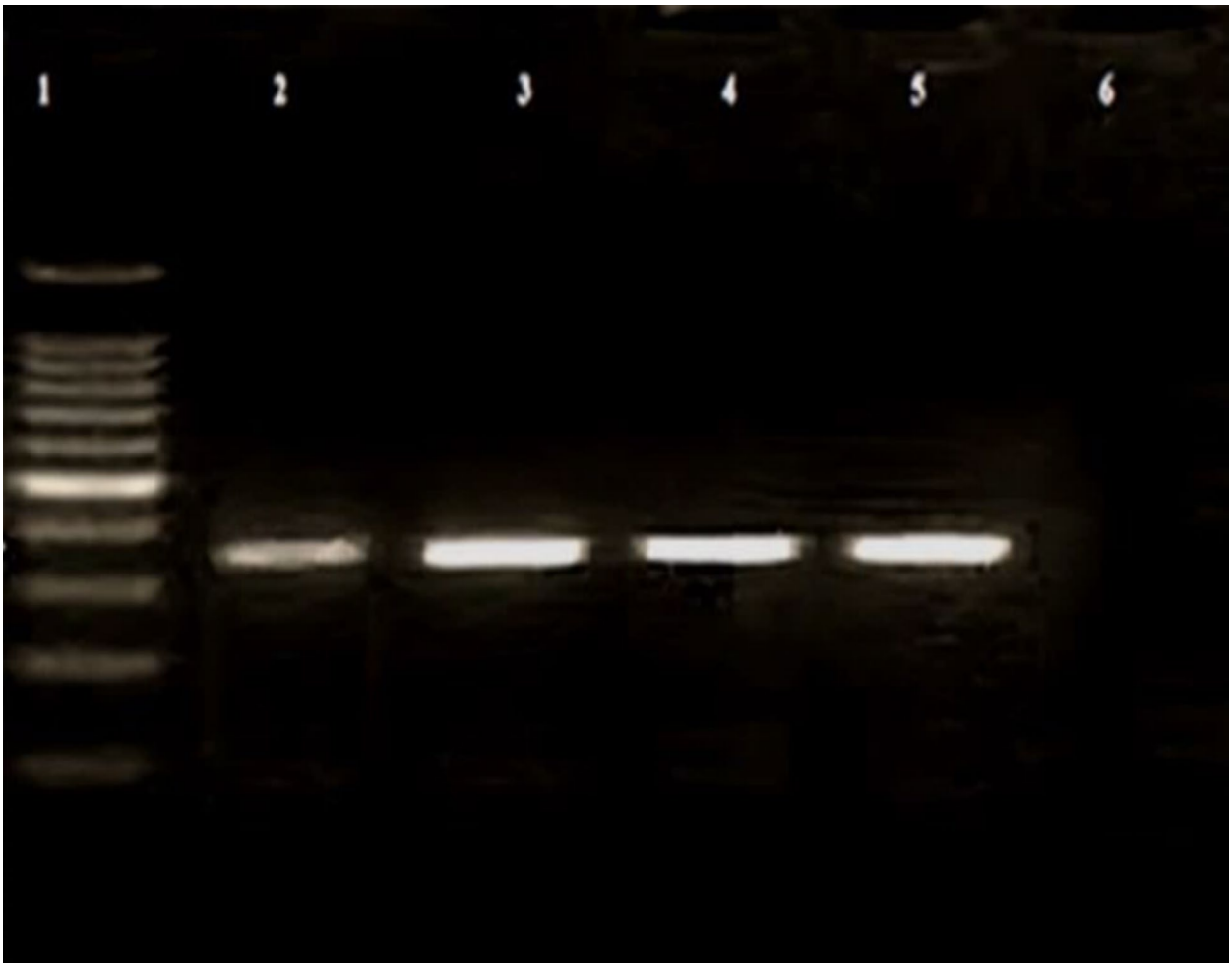


Fig. 3 Agarose gel electrophoresis for the products of the PCR targeting subtype-specific STS gene of *Blastocystis* ST1 at 351 bp. Lane 1: 100-bp DNA molecular weight marker "ladder." Lanes 2–4: positive samples; Lane 5: positive control; Lane 6: negative control (visualized using gel documentation system)

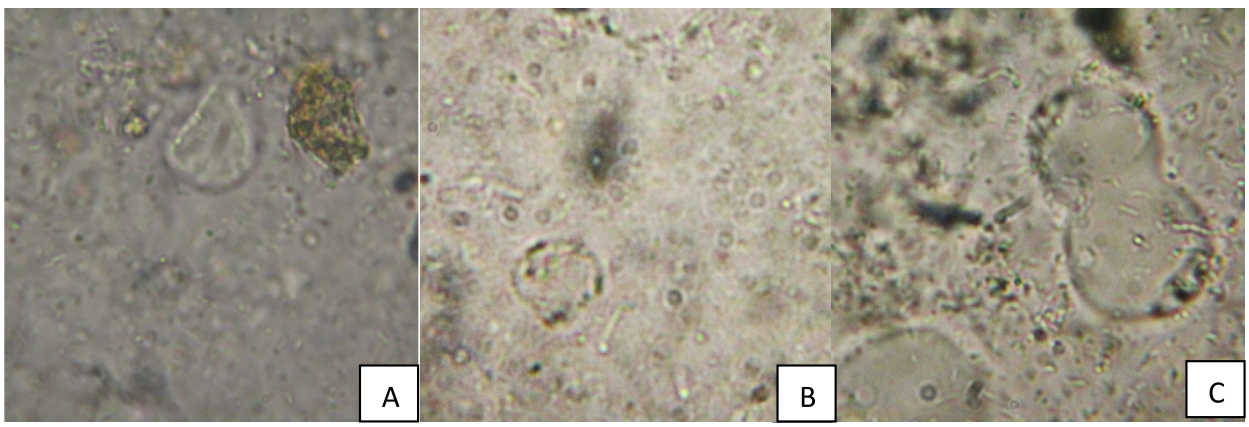


Fig. 4 LM images of a wet mount of cultured *Blastocystis* treated with 100 µg/mL *P. undulata* leaf extract for 24 h reveal outer outline deformity (**A**), a blunt bleb-like bulge at one side (**B**) and maintained multiplication with the nuclear area a bit altered (**C**) [magnification (mag.) × 400]

protrusions (Fig. 4). On increasing exposure time to 72 h, larger *Blastocystis* could be spotted; some appeared swollen inside, pushing peripheral nuclei outwards as if protruding from the surface outline, with a clear staining of the central vacuole on adding Lugol's iodine. Angular edges could be seen sometimes due to swollenness and distortion (Fig. 5).

Flower extract 100 µg/mL concentration

After a 24-h exposure, outline changes could be seen; multiple *Blastocystis* showed single protruding finger-like blebs (Fig. 6). However, 72-h exposure caused an increase in the number of surface blebs in each parasite cell. Blebs appeared shorter with pointed tips, giving an irregular outer outline. Broad bulges, similar in appearance to small daughter cells attached to the original ones, could also be spotted (Fig. 7).

Leaf extract 400 µg/mL concentration

After 24 h, variable nuclear structural changes started to appear in some cells, with some sort of vacuolation or sometimes gathering of swollen nuclei at the parasite cell pole (appearing as cell caps). Outer parasite outline irregularity or even polar extension was observed. However, big-sized cells could be seen with clear nuclear degeneration and interestingly, maintained circular outline sometimes (Fig. 8). On increasing exposure time to 72 h, huge-sized *Blastocystis* could be spotted with small daughter cell-like attachments. Again, nuclear edema and protrusion from the surface were remarkable;

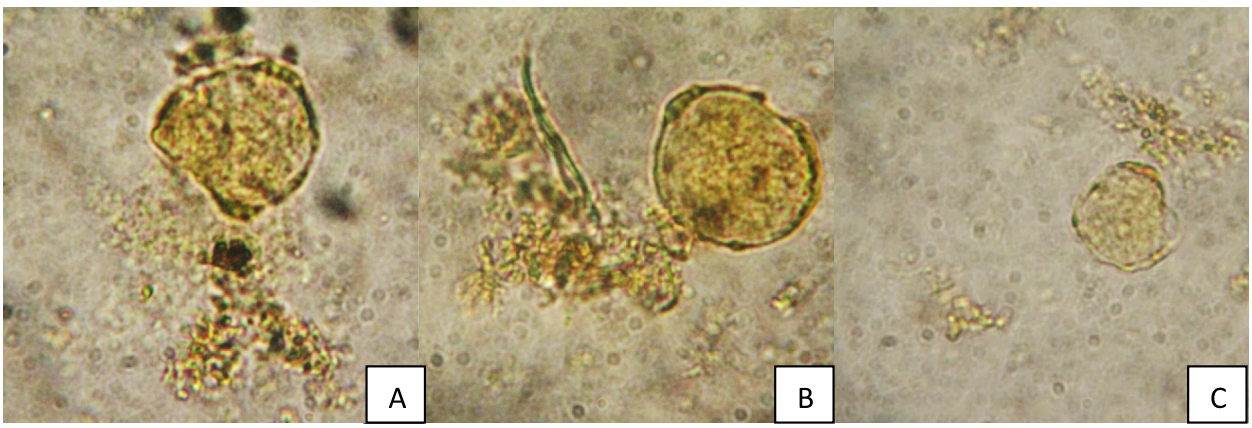


Fig. 5 LM images of cultured *Blastocystis,* stained with Lugol's iodine, treated with 100 µg/mL *P. undulata* leaf extract for 72 h, showing a bigger size, a stained central vacuole and protruding nuclei augmenting outline irregularity. Angular edges and distorted outline (**A**), vacuolar distension (**B**), and outline irregularity as if interrupted (**C**) could also be seen (mag.×400)



Fig. 6 LM images of the wet mount of cultured *Blastocystis,* treated with 100 µg/mL *P. undulata* flower extract for 24 h, showing average-sized *Blastocystis* with outline deep pitting (**A**) and a group of *Blastocystis* with distorted outline and finger-like blebbing repeatedly seen (**B**) (mag.×400)

some parasites showed clear saucer-shaped depressions (Fig. 9).

Flower extract 400 µg/mL concentration

After a 24-h exposure, different-sized *Blastocystis* could be seen with outline deformities. Some showed blunt polar blebs containing degenerated nuclei. Suspicious ruptured forms, corrugated outlines, or large cells could be seen with distorted outlines (Fig. 10). On increasing exposure time to 72 h, balloon-like cells could be seen with these deflated-like depressed sides. Narrow-based finger-like protrusions from the surface were observed.

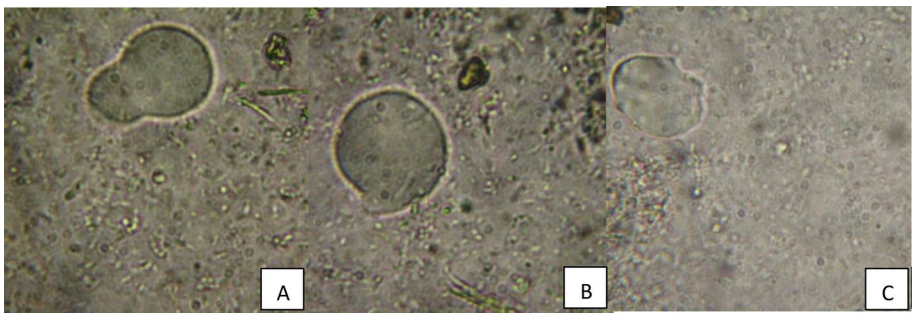


Fig. 7 LM images of wet mount of cultured *Blastocystis*, treated with 100 µg/mL *P. undulata* flower extract for 72 h, showing evident outline deformities: a big lateral bud-like bulge (**A**), lower edge long finger-like extension (**B**) and shorter blebs with pointed tips (**C**) (mag.×400)

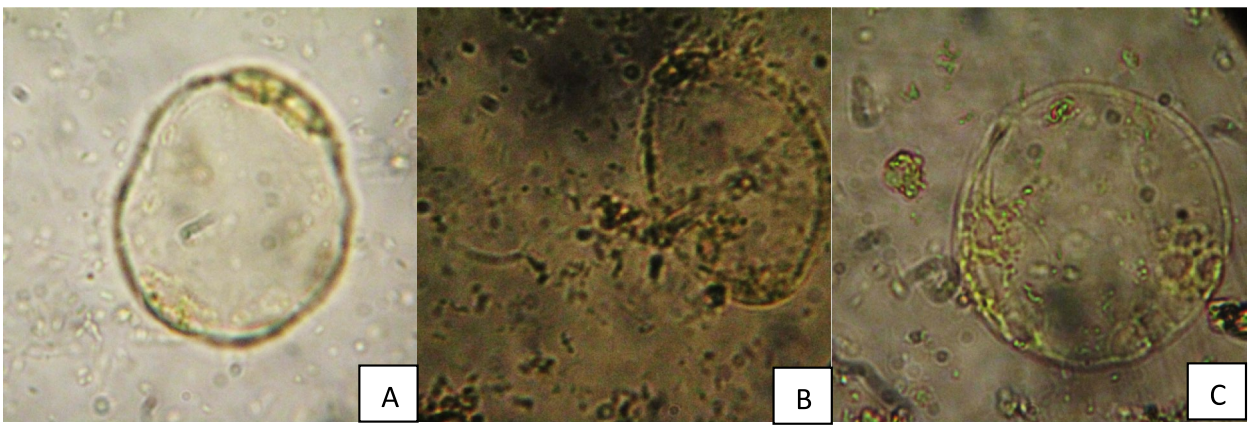


Fig. 8 LM images of cultured *Blastocystis*, stained with Lugol's iodine, treated with 400 µg/mL *P. undulata* leaf extract for 24 h, showing enlarged *Blastocystis*. Outline distortion with a polar gathering of edematous nuclei (**A**), polar expansion with cytoplasm/nuclei polar gathering (**B**) and ambiguous vacuolar degeneration (**C**) could be seen (mag.×400)

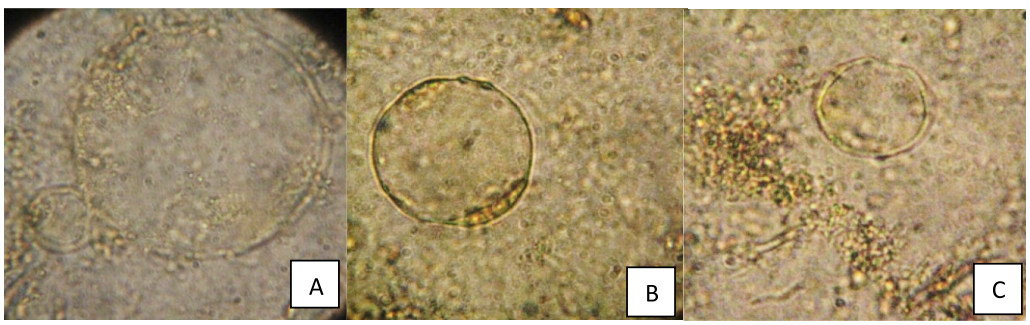


Fig. 9 LM images of cultured *Blastocystis*, stained with Lugol's iodine, treated with 400 µg/mL *P. undulata* leaf extract for 72 h, showing a huge-sized *Blastocystis* with a lateral spherical budding that appears as a twisted lateral bulge (**A**), nuclear edema (lower cap-like gathering) and deforming outer outline (two blebs above) (**B**) and saucer-like depression (**C**) (mag.×400)

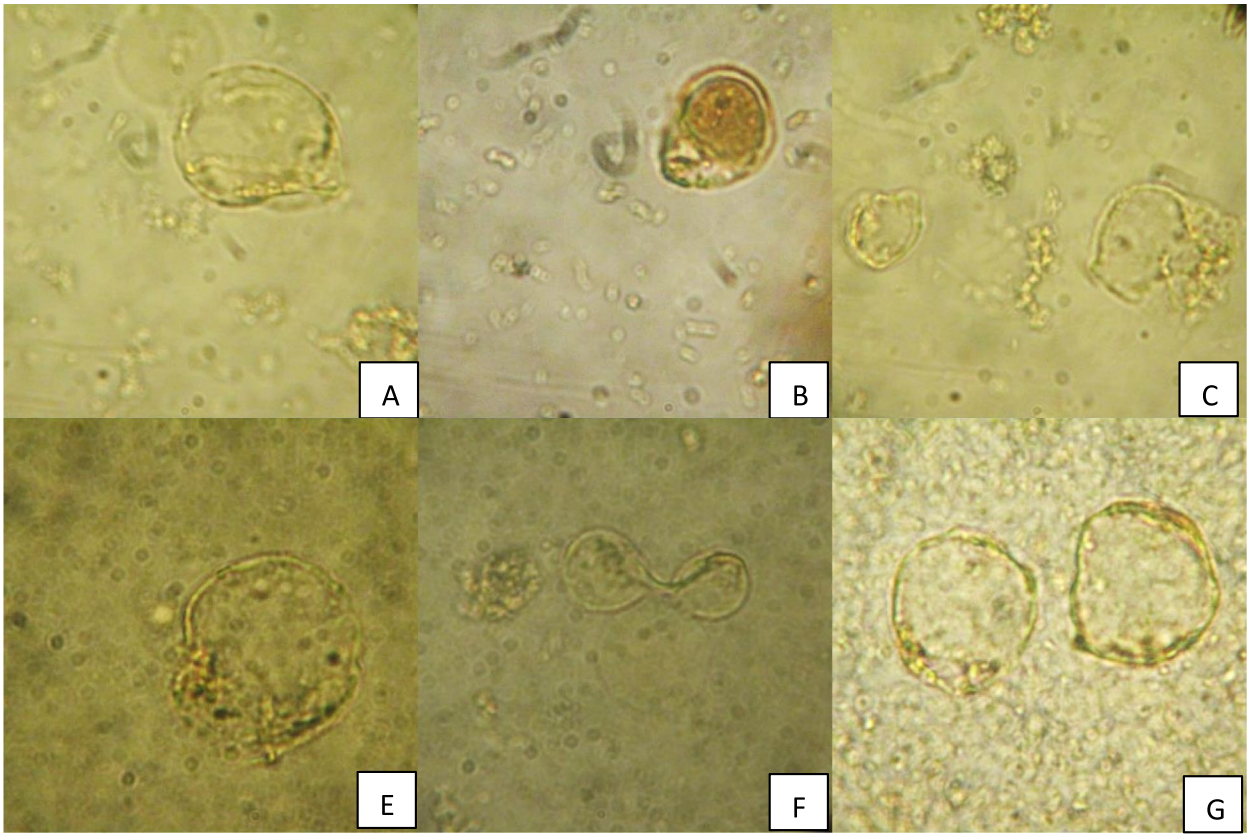


Fig. 10 LM images of cultured *Blastocystis,* stained with Lugol's iodine, treated with 400 µg/mL *P. undulata* flower extract for 24 h, showing lateral undulating bleb with maintained spherical outline (**A**), lateral bulge with distorted nuclear/cytoplasm content and deep staining of vacuolar space (**B**), discontinuous outline with degenerative nuclear/cytoplasmic swelling (**C**), persistent multiplication by division despite atypical cell appearance (**D**), and clearly corrugated outline (**G**) (mag. × 400)

Nuclear changes were evident; some seemed degenerated and swollen, and others seemed flattened and compressed (Fig. 11).

It was remarkable that the longer the exposure time and the higher the administered dose, the larger the size of *Blastocystis* increased. Also, multiplying *Blastocystis* were sometimes observed as dividing forms in treated samples.

Effect of extracts on *Blastocystis* using SEM

It was noticed that the control image of *Blastocystis* using SEM showed a smooth surface (Fig. 12).

Nitazoxanide

After 24-h exposure, many *Blastocystis* maintained normal size and regular spherical outline; a few showed a single bulge or oozing content, and some had shrunken with an overall wrinkled outline. Increasing the exposure time to 72 h, a few *Blastocystis* still maintained their size but revealed some outlined distortion. Many *Blastocystis* were enlarged with wrinkled surfaces; depressions and multiple surface blebs could be observed in some (Fig. 13).

Using a 100 µg/mL concentration

After 24-h exposure, neither leaf nor flower extracts affected the size of most *Blastocystis*; a distorted outline was repeatedly seen; some cells showed singular blebs, generalized surface wrinkling, or a deformed outline. With increasing exposure time up to 72 h, the size of *Blastocystis* tended to enlarge, especially with leaf extract. However, leaf extract induced limited outline changes with few pointed blebs, while with the flower extract, some cells showed angular edges, pointed blebs, bulges, or severe distortion. Suspicious ruptured forms could be observed with both extracts (Figs. 14 and 15).

Using a 400 µg/mL concentration

After 24 h of exposure, larger *Blastocystis* could be observed, especially with flower extract. Angular edges (mostly a representation of wide-based blunt bulges) and medium-sized depressions were observed with leaf

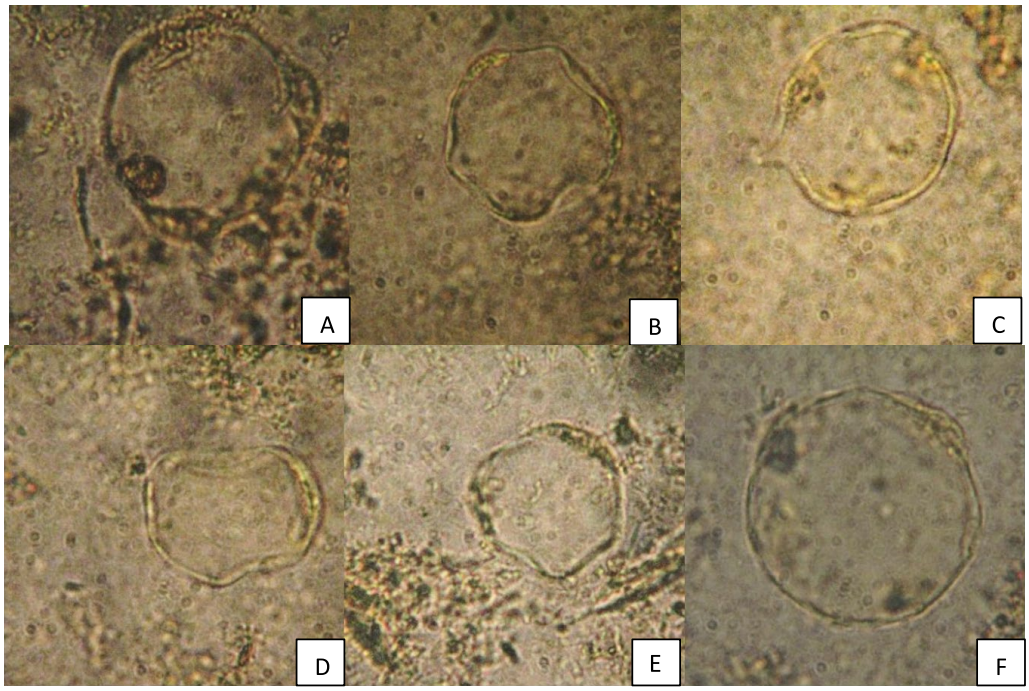


Fig. 11 LM images of cultured *Blastocystis,* stained with Lugol's iodine, treated with 400 µg/mL *P. undulata* flower extract for 72 h, showing enlarged *Blastocystis* with marked vacuolar swollenness (**A**, **C** and **F**). Various nuclear and/or cytoplasmic changes like polar nuclear/cytoplasmic edema (**A** and **E**) and flattened nuclei (**D**) which seem to be due to a primary severe vacuolar distension. Varying forms of outline deformity were revealed as deflated-like broad depressions (**B**, **D** and **E**), and beak-like long narrow-based bulges (**C**) (mag. × 400)

extract. Larger sizes and marked surface wrinkling and blebbing could be seen with the flower extract. After 72 h of exposure, larger-sized *Blastocystis* were a common finding for both extracts, with deep depressions and protruding bulges (narrow finger-like or broad) observed with the leaf extract. Also, huge depressions and rupture-like forms were spotted with flower extract (Figs. 14 and 15).

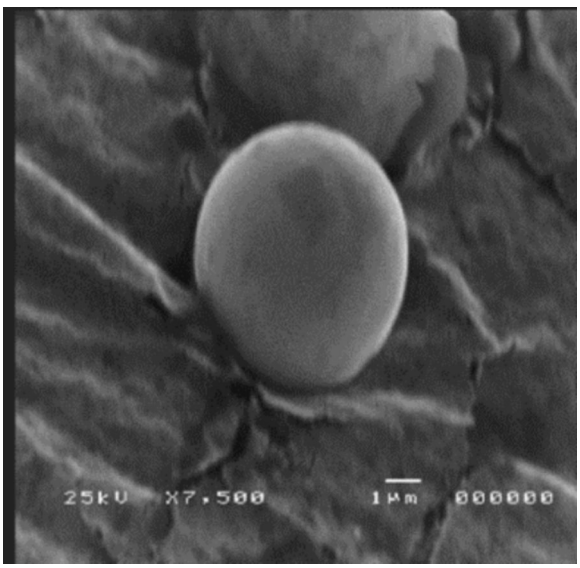


Fig. 12 Shape of *Blastocystis* culture using SEM without adding any extracts or drugs. It has a smooth oval shape with an average size of about 5 µm, well circumscribed with no external contents (mag. X7500)

Plant methanolic extracts metabolite analysis

Metabolites profiling of P. undulata flower and leaf methanolic extracts using GC–MS analysis

Pulicaria undulata flower and leaf methanolic extracts were analyzed via GC–MS post-silylation as part of this study, leading to the identification of 46 metabolites (Table 3) belonging to alcohols, amino acids, fatty acids, flavonoids, organic acids, phenolic compounds, sterols, sugar acids, sugar alcohols, sugars, and terpenoids, as discussed in the next subsections.

Alcohols: Only two glycerol derivatives were identified with a relatively higher percentage in flowers.

Amino acids: Proline is the only amino acid found in flower methanolic extract with a relatively low concentration.

Fatty acids: P. undulata flower and leaf methanolic extracts are rich in saturated and unsaturated fatty acids, with a relatively higher percentage in leaves than flowers (61.25% and 29.81%, respectively), and they are considered the most abundant identified class in leaves. 9, 12-Octadecadienoic acid is the major fatty acid in both

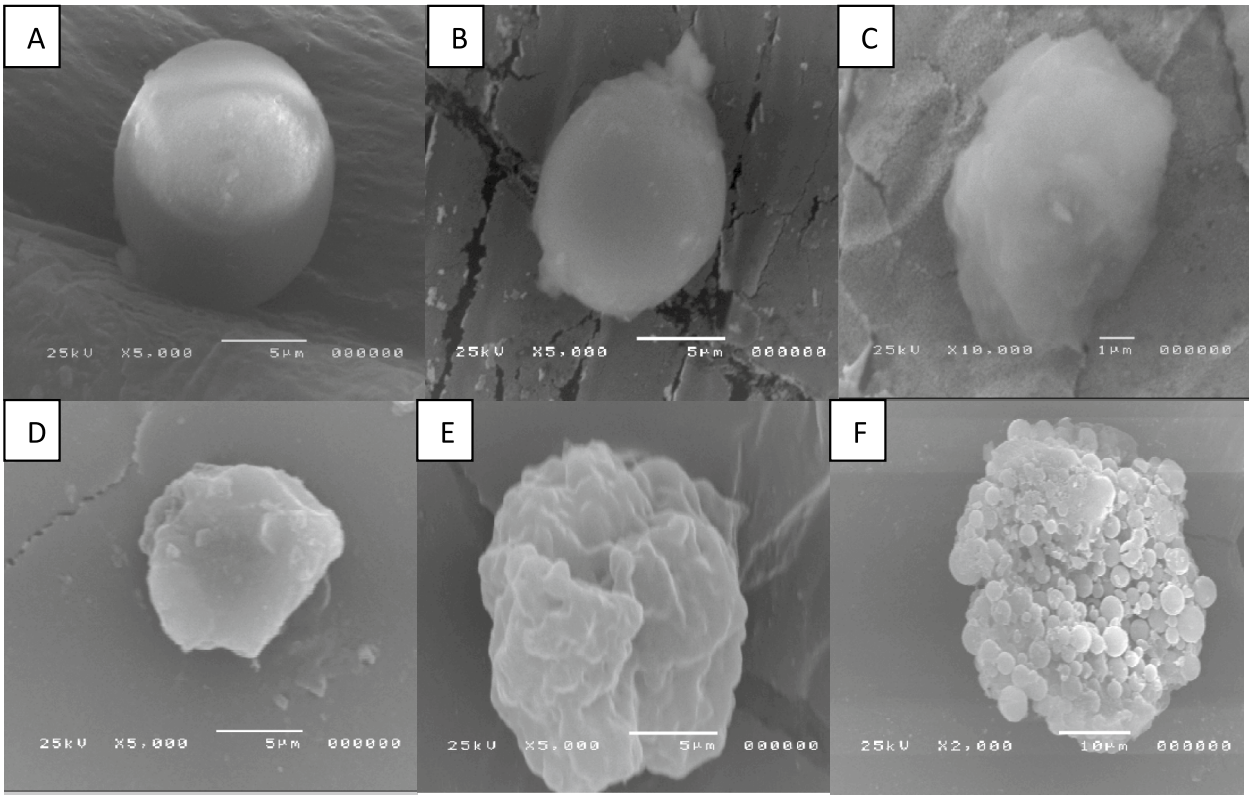


Fig. 13 SEM images of cultured *Blastocystis* treated with NTZ. Changes seen after 24 h included maintained average size and regular shape (**A**), scattered blebs or oozing content (**B**) and size decrease, deformity and external shape crumbling (**C**). In 72 h, polar depressions (**D**), big size with wrinkling and depressions (**E**), and bigger sizes with intense surface blebbing (**F**) were detected [mag. **A**, **B**, **D** and **E** (X5000); **C** (X10000) and **F** (X2000)]

flowers and leaves, with relative percentages of 11.21% and 23.33%, respectively. 9-Octadecenoic acid is present only in leaves with a relatively high percentage (16.03%). Oleic acid was found in both flowers and leaves, with a higher relative percentage observed in the flowers (7.87% compared to 3.12% in the leaves). Palmitic acid is a saturated fatty acid that was identified in flower and leaf methanolic extracts (4.2% and 7.21%, respectively).

Flavonoids: Two flavonoids were detected in the leaf methanolic extract, with a relative percentage of 0.31%.

Organic acids: Oxalic acid is the only organic acid in leaf methanolic extract, with a relative percentage of 5.49%, while quinic acid is the major identified one in flower methanolic extract, with a relative percentage of 3.68%.

Phenolic compounds: Caffeic acid was identified in flower methanolic extract, and 6-methoxy-2-phenethyl-4H-chromen-4-one was detected in leaf methanolic extract with relatively low percentages.

Sterols: Sterols constituted a minor class (0.3–2.5%) of primary metabolites found in *P. undulata* flower methanolic extract only. This class was represented by α1-Sitosterol with a relative percentage of 2.10%.

Sugar acids: There are four sugar acids that were identified only in the flower methanolic extract with a total percentage of 2.58%.

Sugar alcohols: Myo-inositol was detected at a significantly higher percentage in the flower methanolic extract (17.8%) compared to the leaf methanolic extract (1.14%).

Sugars: Sugars represented the most abundant class of metabolites in flowers, with a percentage of 38.63%, and also represented a high percentage in leaves (30.86%). Fructose is the major identified one in flowers, with a percentage of 11.89%.

Terpenoids: Terpenoids, a minor class of metabolites, were represented by only one compound in flower methanolic extract, with a percentage of 1.01%.

According to Table 4 and Fig. 16, the flavonoid content of the tested samples, estimated as quercetin equivalent (QE) (Table 5), was deduced from the following equation:

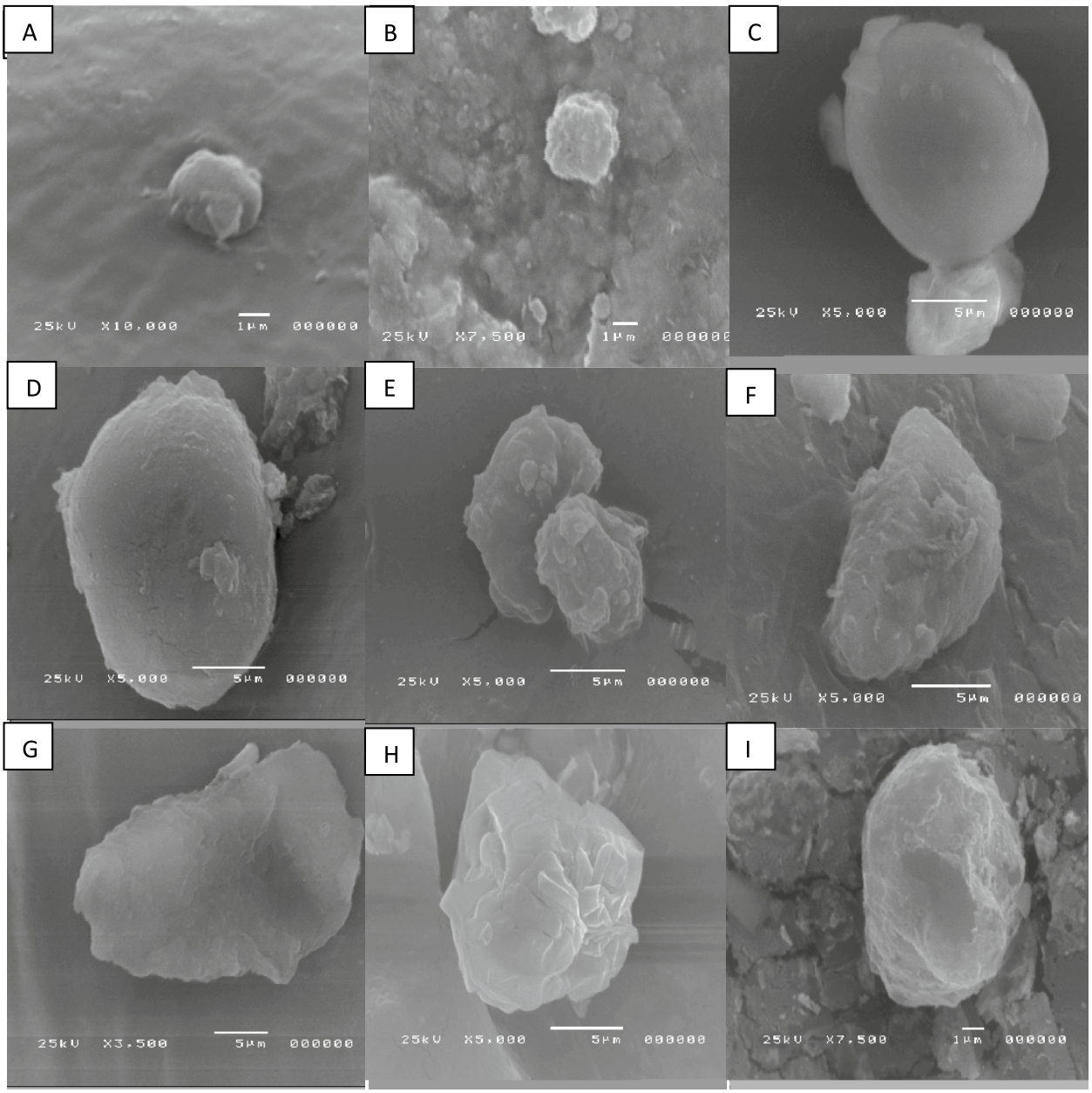


Fig. 14 SEM images of cultured *Blastocystis* treated with *P. undulata* leaf extract showing: (100 µg/mL for 24 h) maintained average size and circular shape with (**A**) single bulge and (**B**) generalized wrinkling; (100 µg/mL for 72 h) increased size, inflated with scattered blebs and some of the contents come out of their walls (**C** and **D**); (400 µg/mL for 24 h) wrinkled surface, variable-sized blebs, with (**E**) polar depressions and (**F**) pointed poles with angular outline deformity; and (400 µg/mL for 72 h)marked deformity with (**G**) size increase and finger-like blebs, (**H**) external shape crumbling, deep broad depressions and (**I**) broad-based bulges. [mag. **C**, **D**, **E**, **F** and **H** (X5000); **A** (X10000); **B** and **I** (X7500) and **G** (X3000)]

$$Y = 0.0083x + 0.1221,\ R^2 = 00.9978,$$

where Y = absorbance, x = corresponding concentration (µg/mL), and R^2 = correlation coefficient.

Flowers methanolic extract exhibited a greatly higher flavonoid concentration than leaves.

Discussion

Pulicaria undulata, often referred to as "Gethgath," is one of the aromatic flowering plants and a member of the sunflower family. Its volatile compounds contribute to its widespread usage in herbal teas, perfumery, or as a natural insect repellent. The essential oils in *P. undulata* have been extensively studied, revealing key volatile

components such as carvotanacetone, carvacrol, borneol, linalool, and camphor. Significant differences were observed in the composition, yield, and content of these oils depending on the geographic origin of the plants. These variations highlight the influence of location on the chemical profile of *P. undulata* [43, 44]. Along with isoflavone glycosides, *P. undulata* has also been shown to contain a flavonoid class of chemicals, primarily of the kaempferol and quercetin glycosidic origins [45]**.** Steroids, triterpenes, tannins, coumarins, and saponins have been identified as secondary metabolites. Traditionally, *P. undulata* has been used by locally working herbalists and nomadic communities in Sudan, especially in the north, as a natural remedy for a range of ailments, including epilepsy, influenza, the common cold, gastrointestinal issues, back pain, joint inflammation, and other inflammatory conditions [46].

In the present study, leaf and flower methanolic extracts of *P. undulata* demonstrated a significant impact on the ultrastructure of *Blastocystis* cultured in vitro, as well as an inhibitory effect on colonic motility. As this is the first study to examine how *P. undulata* methanolic extracts affect *Blastocystis* and colonic motility, the detected structural alterations in the parasite and the reduction in colonic motility may be attributed to the phenolic-rich essential oils and their antioxidant properties [47]**.**

Microscopic study using both light microscope and scanning electron microscope SEM indicated that both NTZ and *P. undulata* extracts induced more noticeable morphological alterations with higher doses and prolonged exposure time. Similarly, a published study [24] recorded an increasing effect of NTZ on *Blastocystis* observed in vitro with higher doses and longer exposure times. Similarly, other in vitro studies demonstrated the influence of various plant extracts on *Blastocystis* [48–50]**,** revealing dose- and exposure time-dependent effects. A key finding was the increase in *Blastocystis* size with higher doses and longer exposure durations. This could suggest that the therapeutics are effective, potentially by causing parasite swelling and edema linked to degenerative changes, and/or by lowering competitive inhibition as parasites are destroyed. The emergence of larger forms may thus reflect a decrease in parasite load.

In agreement, a similar work reported an increased size of parasites like Toxoplasma in a SEM study after therapeutics administration [51]. Also, another study [52] suggested that morphological alteration can affect protozoal reproduction ability with a subsequent decrease in numbers. Besides, others explained that compounds inducing parasite morphological changes can affect their DNA synthesis [53]. This supports the current study finding of repeatedly encountering nuclear/cytoplasmic changes in *Blastocystis* with higher doses and longer exposure times, suggesting different degenerative states [54, 55]**.** On the contrary, giant forms in culture were reported [56]**.** However, in the current study, they were minimal in untreated culture and with low-dose therapy, but obvious with higher therapeutic doses and longer exposure times of plant extracts.

Furthermore, several studies have investigated the impact of *P. undulata* on various parasites, with results that are consistent with the findings of the current study. For instance, *P. undulata* extract was reported to exhibit a significant dose- and time-dependent increase in in vitro *Giardia* cyst mortality [34]. The authors concluded that aqueous extracts of *P. undulata* showed efficacy against Giardia in both in vitro and in vivo studies, indicating its potential as a natural therapeutic alternative to metronidazole.

Likewise, another research [57] assessed the effectiveness of *P. undulata* extracts against both L*eishmania major* as well as *Toxoplasma gondii*. Petroleum ether, n-butanol, ethyl acetate, chloroform, and water were used to fractionate *P. undulata* after its extraction using methanol. In vitro tests of the resultant fractions were conducted against the amastigote stage of *Toxoplasma gondii* and promastigotes of *Leishmania major*. Additionally, Vero cell line was used to assess cytotoxicity. The methanolic extract showed notable anti-toxoplasmal activity and was the most effective fraction against both promastigotes and amastigotes of *Leishmania major*. The study concluded that *P. undulata*'s chloroform fraction holds high potential for isolating active compounds with anti-toxoplasmal and anti-leishmanial properties.

(See figure on next page.)

Fig. 15 SEM images of cultured *Blastocystis* treated with *P. undulata* flower extract showing: (100 µg\ml for 24 h) maintained average size and circular shape with (**A)** surfaced indentations and (**B)** wavy outline with several surface bulges; (100 µg\ml for 72 h) (**C)** average size, deformed with pointed protrusion and several broad bulges and (**D)** huge enlargement with frank polar deflation and crumbling; (400 µg\ml for 24 h) severely wrinkled surface and multiple pointed blebs (**E** and **F)**; and (400 µg\ml for 72 h) marked deformity and corrugated surface with (**G)** size increase and a ruptured-like appearance and (**H)** severe inflation with polar deflation (broad saucer- like depression) [mag. **A**, **B**, **C** and **E** (X7500); **F** and **H** (X5000); **A** (X10000); and **D** (X750)]

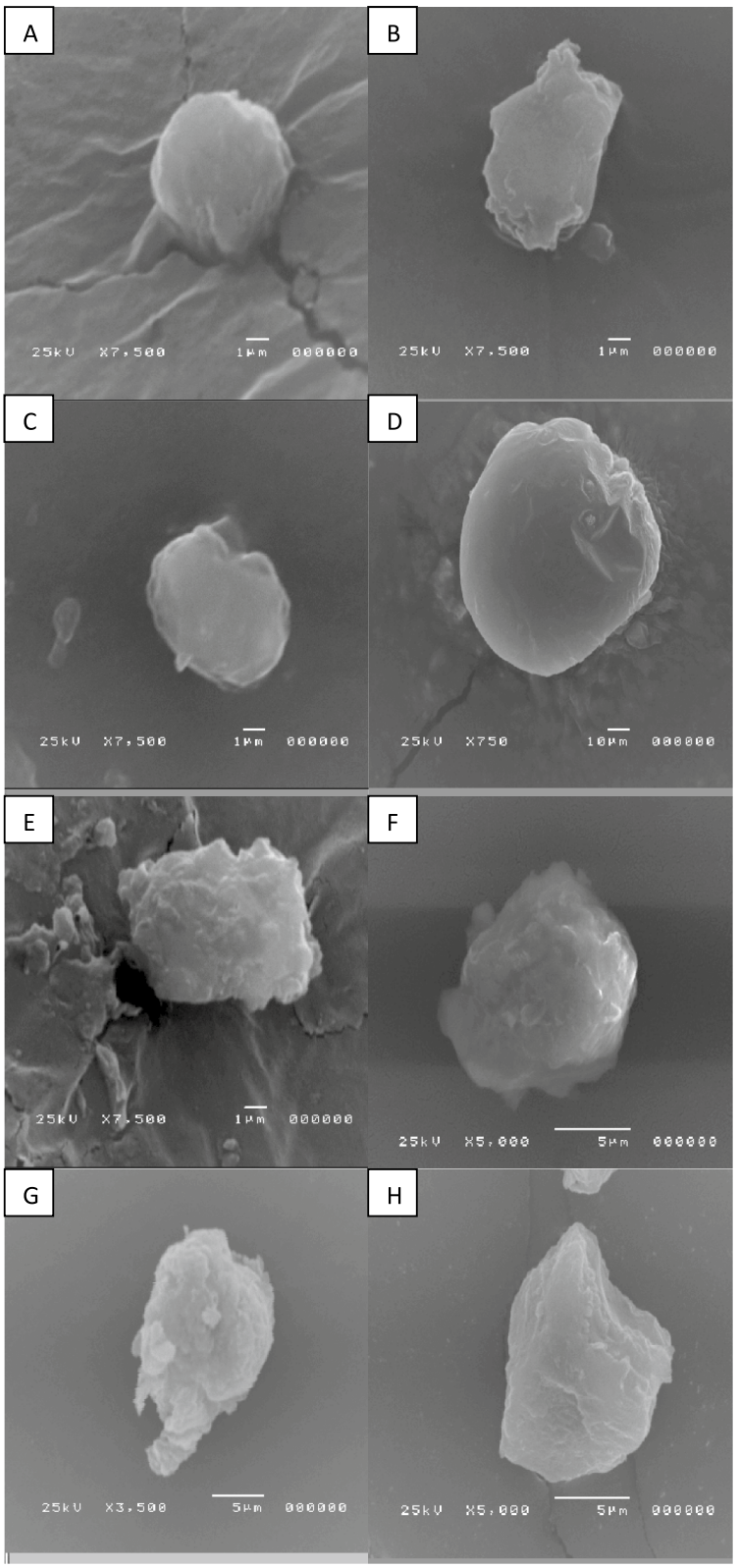


Fig. 15 (See legend on previous page.)

Table 3 Primary metabolite analysis using GC-MS from *P. undulata* flower and leaf methanolic extracts expressed as an average relative percentile

RI	Mwt	Compound name	Flower methanolic extract	Leaf methanolic extract
Alcohol				
1289	308	Glycerol, 3TMS derivative	1.31	0.22
2180	686	2-O-Glycerol-α-d-galactopyranoside, hexa-TMS	0.13	
Total alcohols			1.44	0.22
Amino acids/Nitrogenous compounds				
1305	259	L-Proline, 2TMS derivative	0.61	
Total amino acids			0.61	
Fatty acids				
1655	272	Dodecanoic acid, TMS derivative		5.14
2050	328	Palmitic Acid, TMS derivative	4.20	7.21
2212	352	9,12-Octadecadienoic acid (Z,Z)-, TMS derivative	11.21	23.33
2218	354	Oleic Acid, (Z)-, TMS derivative	7.87	3.12
2222	354	9-Octadecenoic acid, (E)-, TMS derivative		16.03
2232	354	13-Octadecenoic acid, (Z)-, TMS derivative	1.81	
2246	356	Stearic acid, TMS derivative	3.09	4.14
2304	352	9(E),11(E)-Conjugated linoleic acid, trimethylsilyl ester	0.20	
2447	384	Arachidic acid, TMS derivative		0.03
2607	474	1-Monopalmitin, 2TMS derivative	0.68	1.38
2644	412	Behenic acid, TMS derivative	0.75	0.84
2785	440	Heneicosanoic acid, TBDMS derivative		0.03
Total fatty acids			29.81	61.25
Flavonoids				
2881	664	cis-Dihydroquercetin, 5-OTMS		0.23
2368	340	3-Hydroxy-2′-methoxyflavone, trimethylsilyl ether		0.08
Total flavonoids				0.31
Organic acids				
793	234	Oxalic acid, 2TMS derivative		5.49
1098	131	Oxalic acid mono-(N-dimethyl)-amide, methyl ester	0.33	
1344	322	Glyceric acid, 3TMS derivative	0.16	
1497	350	Malic acid, 3TMS derivative	0.64	
1851	552	Quinic acid (5TMS)	3.68	
Total organic acids			4.81	5.49
Phenolic compounds				
2155	396	Caffeic acid, 3TMS derivative	0.64	
2613	280	6-Methoxy-2-phenethyl-4H-chromen-4-one		0.72
Total phenolic compounds			0.64	0.72
Sterols				
3313	426	α1-Sitosterol	2.10	
Total sterols			2.10	
Sugar acids				
1623	364	Arabinonic acid, 2,3,5-tris-O-(trimethylsilyl)-, γ-lactone, d-	0.11	
1925	466	Galactonic acid, γ-lactone, 4TMS derivative	0.85	
1932	466	Gluonic acid, γ-lactone, 4TMS derivative	1.56	
2012	466	Mannonic acid, γ-lactone, 4TMS derivative	0.06	
Total sugar acids			2.58	
Sugar alcohols				
1980	614	D-Glucitol, 6TMS derivative	0.58	

Table 3 (continued)

RI	Mwt	Compound name	Flower methanolic extract	Leaf methanolic extract
2129	612	Myo-Inositol, 6TMS derivative	17.81	1.14
Total sugar alcohols			18.39	1.14
Sugars				
1618	452	L-(+)-Rhamnopyranose, 4TMS derivative	0.31	
1657	452	L-Rhamnose, 4TMS derivative	0.34	7.53
1748	438	Arabinose, 4TMS derivative	0.23	
1792	540	D-(-)-Fructofuranose, pentakis(trimethylsilyl) ether (isomer 1)	5.61	11.62
1799	540	D-Psicofuranose, pentakis(trimethylsilyl) ether (isomer 1)	0.09	
1800	540	D-(-)-Fructofuranose, pentakis(trimethylsilyl) ether (isomer 2)	10.98	
1841	540	D-(+)-Talofuranose, pentakis(trimethylsilyl) ether (isomer 2)	0.30	
1844	540	D-Mannose, 5TMS derivative		2.12
1852	540	β-D-Galactofuranose, 1,2,3,5,6-pentakis-O-(trimethylsilyl)-	1.93	3.84
1867	540	D-Fructose, 5TMS derivative	11.89	0.42
1875	569	D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	0.22	
1926	540	D-Glucose, 5TMS derivative	6.37	3.40
2649	918	Maltose, 8TMS derivative	0.02	
2712	918	Sucrose, 8TMS derivative	0.34	1.93
Total sugars			38.63	30.86
Sugars				
3155	566	Andrographolide, tri-(trimethylsilyl)-	1.01	
Total terpenoids			1.01	

RI: Kovats retention index (calculated relative to n- alkanes, C8–C30). Compound identification was based on both mass spectral matching (NISTlibraries) and RI agreement with literature values

Table 4 Determination of total flavonoid content using different concentrations of quercetin standard

Concentration (µg/mL)	Absorbance *
10	0.21005
20	0.30425
40	0.4339
80	0.7652
120	1.12965

*Average of two determinations

Besides, the anti-inflammatory and antitoxic effect of *P. undulata* was established in previous research [58], showing that the genus *Pulicaria* has a cytotoxic effect, but further research is required to examine the active compounds and their potential development into new anticancer medications.

In the current study, plant extracts demonstrated a noticeable effect on colonic motility, aligning with previous research indicating that components of *P. undulata* possess spasmolytic properties [59]. The antispasmodic effect observed in the present study was introduced by a noticeable reduction in colonic contractility and diminished amplitude of response to the cholinomimetic agent methacholine. The spasmolytic effect was particularly prominent with the leaf extract. These preliminary findings warrant further investigation to recognize the exact pharmacologically active compounds inducing this inhibitory action. On the other hand, a previous study [22] assessed the chemical composition and protective role of *P. undulata* against gastric ulcers induced by ethanol in rats and they concluded that *P. undulata* showed a promising anti- ulcerative protective effect. They also recommended performing further studies to identify the biologically active compounds responsible for their protective effect against chemically induced gastric ulcers.

In the current study, *P. undulata* flower and leaf methanolic extracts were analyzed *via* GC-MS post-silylation. Forty six metabolites were identified belonging to alcohols, amino acids, fatty acids, flavonoids, organic acids, phenolic compounds, sterols, sugar acids, sugar alcohols, sugars, and terpenoids. Flavonoids were present in high concentrations in both flower and leaf methanolic extracts, with higher concentrations in flower methanolic extract, which could be attributed to their effective improvement in IBS symptoms [60, 61]. Also, fatty acids

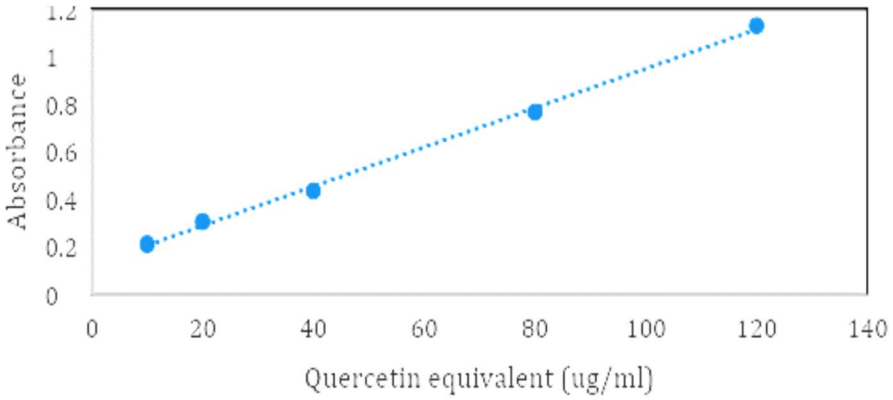


Fig. 16 Standard calibration curve of quercetin

Table 5 Total flavonoid content (TFC), estimated as µg quercetin equivalent (QE)/mg dried extract, of dried flower or leaf extracts:

Sample	Concentration (µg QE/mg dried extract)
Leaves	11.737
Flowers	26.948

represented the most abundant metabolites in leaf methanolic extract, which could be the major effector of the significant antiparasitic activity of leaves [62, 63].

Several metabolites, detected in flower and leaf methanolic extracts used in the current study including fatty acids (palmitic, oleic, linoleic acids), sterols (β-sitosterol), and sugars (glucose and fructose), have also been consistently reported in previous studies on *P. undulata* and related *Pulicaria* species[63], supporting the reliability of our current GC–MS profiling.

The detection of phenolic compounds and flavonoids in our GC–MS analysis is consistent with earlier phytochemical studies on *P. undulata*, which also reported the presence of these metabolite classes [64]. This agreement, together with our experimentally measured total flavonoid content, confirms that phenolics and flavonoids are important constituents of *P. undulata* extracts.

In the same context, traditional applications, biological activity, and phytochemistry of *Pulicaria* species found in Qatar were critically reviewed [63], concerning the plant's pharmacological components. They found that these species' essential oils, crude extracts, their fractions, and sequestered compounds exhibit a variety of biological and pharmacological reactions, primarily *in-vitro*. Several characteristic compounds are mentioned for each species and may be used as chemical identifiers for defining them; most of the traditional claims of these species have been confirmed by recent scientific studies. For their possible application as therapeutic agents, they suggested that more thorough in vivo clinical studies be conducted.

Conclusion

This study represented a step toward identifying the potential use of an eastern medicinal plant *P. undulata,* in managing and alleviating both the cause and symptoms of IBS while attempting to point out its methanolic extracts metabolites and their possible influences. The current results confirmed a clear spasmolytic activity and inhibition of colonic contractility by *P. undulata* methanolic extracts that could be related to the high flavonoid content detected. Again, *P. undulata* methanolic extracts revealed marked deforming morphological and ultrastructural changes on *Blastocystis* in vitro, a widely spread intestinal parasite whose increased numbers are currently implicated in IBS symptoms. These changes were detected by light and scanning electron microscopy and were rather comparable to those induced by NTZ. GC–MS post-silylation analysis identified 46 different metabolites in *P. undulata* flower and leaf methanolic extracts. Flowers methanolic extract exhibited a greatly higher flavonoid concentration than leaves. Further studies are recommended to isolate the effective compounds and investigate their therapeutic dosing and related undesirable effects or contraindications.

Abbreviations

bp	Base pairs
C	Carbon
$CaCl_2$	Calcium chloride
CBT	Cognitive behavioral therapy
COVID-19	Coronavirus disease 2019

DMAs	Disease-modifying agents
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetraacetic acid
FGIDs	Functional gastrointestinal diseases
FODMAP	Fructans, oligosaccharides, disaccharides, monosaccharides, and polyols
GC/MS	Gas chromatography-mass spectrometry
GC–MS-QP	Gas chromatograph mass spectrometer that uses a quadrupole (QP) mass analyzer
i.d.	Inner diameter
IACUC	Institutional Animal Care and Use Committee
IBD	Inflammatory bowel disease
IBS	Inflammatory bowel disease
IBS-C	Constipation-predominant
IBS-D	Diarrhea-predominant
IBS-M	Mixed stool pattern
IBS-U	Amorphous type
IL	Interleukin
JAK	Janus kinase inhibitor
KCl	Potassium chloride
KI	Kovats index
LM	Light microscope
M	Molar
mag.	Magnification
$MgCl_2$	Magnesium dichloride
mM	Millimolar
Mwt	Molecular weight
NaCl	Sodium chloride
$NaH_2 PO_4$	Sodium dihydrogen phosphate
$NaHCO_3$	Sodium bicarbonate
NIST	National institute of standards and technology
NTZ	Nitazoxanide (nanazoxid)
OTMS	Octadecyltrimethoxysilane
P. undulata	*Pulicaria undulata*
PCR	Polymerase chain reaction
PCS	Post-COVID-19 syndrome
QE	Quercetin equivalent
RI	Retention index
S1P	Sphingosine 1-phosphate
SEM	Scanning electron microscope
SSU rDNA	Small-subunit ribosomal RNA gene
ST	Subtype
STS	Sequence-tagged-site
TBDMS	Tert-butyldimethylsilyl
TF	Total flavonoid
TFC	Total flavonoid content
TMS	Tetramethylsilane
UPLC-ESIQ-TOF	Qualitative ultra-performance liquid chromatography with the ionization and mass analysis of Electrospray Ionization and Quadrupole-Time of Flight
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight by weight
5-HT3	Serotonin type 3 (5-hydroxytryptamine)

Author contributions
EMAR, NZB, and IAH contributed to the study conception and design. EMAR, NZB, IAH, and MSS were responsible for material preparation, methodology, data collection, analysis, writing the manuscript, and preparing the figures. EMAR, NZB, and EKR were responsible for revising and editing the manuscript. All authors commented on previous versions of the manuscript, then read, and approved the final manuscript.

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Data availability
Mostly all generated or analyzed data are included in the written manuscript.

Declarations

Ethics approval and consent to participate
Handling and animal cares were performed according to guidelines of the ethical committees of Cairo University -Institutional Animal Care and Use Committee (CU-IACUC) number **CU-III- F- 55- 24]** and complied with the National Research Council's Guide for the Care and Use of Laboratory Animals for all experiments.

Consent for publication
The content of the manuscript has not been published or submitted for publication elsewhere.

Competing interests
No financial or non-financial competing interests have been declared by the authors of the current work.

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