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PHYTOCHEMICAL PROFILING, CYTOTOXIC, ANTIOXIDANT AND IMMUNOSTIMULATORY EFFECTS OF *CYPERUS ROTUNDUS*: EVIDENCE-BASED VALIDATION OF A TRADITIONAL MEDICINAL PLANT

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ABSTRACT

Background: *Cyperus rotundus*, a traditional medicinal plant, has been extensively studied for its phytochemical composition and therapeutic properties, including its anticancer and anti-inflammatory activities. *Cyperus rotundus* is used in traditional medicinal systems for the treatment of diarrhea, dysentery, dysmenorrhea, and menstrual irregularities. This study aimed to investigate the phytochemical profile and cytotoxic, antioxidant, and immunostimulatory effects of *Cyperus rotundus* extract.

Methods: Ethanol extract (CRE) and subsequent dichloromethane (CRD) and aqueous (CRA) fractions were prepared from *Cyperus rotundus* using the Soxhlet method. Various methods have been used to screen phytochemicals. The antioxidant capacity of the extracts was assessed using the DPPH assay. The cytotoxic potential of the extracts was evaluated in Hep2 cells using an MTT assay. The immunomodulatory effects of *Cyperus rotundus* were examined using flow cytometry-based surface immunophenotyping, focusing on the activation markers CD80 and CD86 as well as Intracellular Cytokine Staining for Interleukin-6 and Tumor Necrosis Factor-α.

Results: Phytochemical screening revealed the presence of alkaloids, phenolics, saponins, glycosides, steroids, phytosterols, flavonoids, and carbohydrates in the extracts. The aqueous extract showed minimal cytotoxicity on Hep2 cells with an IC50 of 189.8 µg/mL, while the ethanolic and dichloromethane extracts had IC50 values of 372.5 µg/mL and 263.7 µg/mL, respectively. The aqueous extract exhibited the highest DPPH radical scavenging activity (IC50 = 310.8 µg/mL), followed by the ethanolic extract (IC50 = 591.2 µg/mL). Gas chromatography-mass spectrometry (GC-MS) analysis of the aqueous extract identified forty-three compounds. Flow cytometry analysis demonstrated that the aqueous extract significantly enhanced the activation of monocytes, monocyte-derived dendritic cells (MoDCs), and plasmacytoid dendritic cells (pDCs) by upregulating the expression of the costimulatory molecules CD80 and CD86. Intracellular cytokine staining revealed increased production of IL-6 and TNF-α in monocytes upon treatment with the aqueous extract.

Conclusion: These findings validate the ethnomedicinal use of *Cyperus rotundus* as an immunomodulator and highlight its potential as a safe phytomedicine for managing oxidative stress and immune dysfunction

KEYWORDS: *Cyperus rotundus*, Immunomodulation, Cytotoxicity, GC-MS, Antioxidant, Phytochemicals, Herbal Medicine, monocyte-derived dendritic cells and plasmacytoid dendritic cells

INTRODUCTION

Immunomodulators modify the immune system responses by enhancing or suppressing immune activity to restore balance. They regulate immune cells and signalling molecules through targeted actions, such as monoclonal antibodies, or broad effects, such as corticosteroids(1). Therapeutic applications include transplantation immunosuppression to prevent organ rejection and the treatment of autoimmune diseases(2). In cancer therapy, they function as checkpoint inhibitors or CAR T-cell therapy, boosting immunity against chronic infections and serving as prophylaxis for immunocompromised

patients. Immunomodulators are vital tools in modern medicine, enabling the immune system to treat diseases, such as infections and cancers. Their development continues to transform therapeutic strategies (3).

Cyperus rotundus, or nut grass, a perennial plant of the *Cyperaceae* family, has rhizomes and tubers with medicinal properties. Native to India, it is naturalized in Africa, America, Asia, and Europe (4). *Cyperus rotundus* is valued in traditional medicine for the treatment of diarrhea, dysentery, dysmenorrhea, and menstrual irregularities owing to its antimicrobial and antispasmodic effects. The plant exhibits analgesic and inhibitory effects against pathogens, demonstrating neuroprotective and neuromodulatory properties (Balkrishna et al., 2020). It exhibits anticancer activity by inhibiting proliferation and inducing apoptosis in cancer cells, whereas its antioxidant properties contribute to cardiovascular protection. The phytochemical profile of the plant includes essential oils, flavonoids, alkaloids, and terpenoids, all of which contribute to its biological activity. Various sesquiterpenes, iridoid glycosides, and phenolics have been isolated from *Cyperus rotundus* (5).

Given the crucial role of immunomodulators in modern medicine, this study focused on *Cyperus rotundus*, which is known for its immunomodulatory properties. We aimed to bridge the gap between traditional usage and evidence-based validation through comprehensive phytochemical profiling and assessment of its cytotoxic, antioxidant, and immunostimulatory effects.

MATERIALS AND METHODS

Plant Collection and Identification

The whole plant of *Cyperus rotundus* was collected from a local market in Theni, Tamil Nadu, and the plant was identified at the Department of Botany, Madurai Kamaraj University. A voucher specimen was deposited in the department, and the characteristics of this plant were studied and matched with the available literature. The collected fresh plant material was air-dried, ground to a fine powder, and stored in airtight containers for further analysis.

Extraction

To extract *Cyperus rotundus* using the Soxhlet method, the tubers were first cleaned, shade-dried, and finely powdered. Approximately 100 g of the powder was loaded into a cellulose thimble and placed in a Soxhlet extractor. Three different solvents, distilled water (aqueous), ethanol, and dichloromethane, were used for sequential extractions to target a broad spectrum of phytoconstituents ranging from polar to non-polar.

For each solvent, 1500mL is added to a round-bottom flask below the extractor. The assembly was heated to maintain the solvent at its boiling point, allowing for continuous reflux and siphoning for over 6h, which ensured efficient and exhaustive extraction. Water, ethanol, and dichloromethane were used at 100 °C, 78 °C, and 40 °C, respectively. After completion, the extracts were collected and concentrated under reduced pressure using a rotary evaporator to preserve the thermolabile components. The concentrated extracts were stored at 4 °C in airtight containers (6).

Phytochemical Screening

The phytochemical analysis of *Cyperus rotundus* involves a comprehensive qualitative evaluation of its bioactive constituents from the prepared extracts using a series of standardized qualitative phytochemical tests to detect the presence of primary metabolites. These tests include the Ferric Chloride Test for phenolics and tannins, alkaline reagent test for flavonoids, foam test for saponins, Liebermann-Burchard test for steroids and terpenoids, Borntrager's test for glycosides, Molisch's and Benedict's tests for carbohydrates, and Liebermann-Burchard's test for steroids and terpenoids (7).

Cell viability by MTT assay

The MTT assay was used to assess the cytotoxicity and viability of Hep2 cell lines after treatment with aqueous, ethanol, and dichloromethane (DCM) extracts of *Cyperus rotundus*. Hep2 cells were seeded in 96-well plates at a density of 10,000 cells/well in complete DMEM and incubated at 37 °C with 5% CO_2 for 24h to allow attachment. Cells were then exposed to varying concentrations (62.5–1000µg/mL) of each plant extract, prepared in sterile solvent, and incubated for a further 24 h. For each concentration, untreated cells served as negative controls, and wells with solvent alone were used as vehicle controls.

After treatment, 20µL µL MTT solution (5 mg/mL in PBS) was added to each well, and the plates were incubated for 4h at 37 °C. The resulting purple formazan crystals formed by metabolically active cells were dissolved by adding 100 µL of DMSO. The absorbance was measured at 570 nm using a microplate reader. Cell viability (%) was calculated relative to that of the control. Each assay was performed in triplicate, and the data are expressed as mean±SD. IC_{50} values were determined by plotting dose–response curves (8).

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay is a widely adopted method for evaluating the antioxidant capacity of plant extracts. To assess the antioxidant properties of *Cyperus rotundus*, both extracts were serially diluted in their respective solvents to obtain varying concentrations (125–1000µg/mL). An aliquot (1mL) of each extract was mixed with 1mL of a 0.1mM DPPH solution in methanol and incubated in the dark at room temperature for 30 min to allow reaction with free radicals.

The reduction in DPPH radicals, visible as a decrease in purple color intensity, was measured by recording absorbance at 517 nm using a UV-visible spectrophotometer. A solution of DPPH and the solvent alone served as the control, whereas ascorbic acid was used as a standard. The percentage DPPH radical scavenging activity was calculated using the following formula:

Radical scavenging (%) = (Abs Control−Abs sample)/ Abs control x 100

Each assay was performed in triplicate, and the results are expressed as mean±SD. IC50 values were determined from the dose-response curve (9).

Gas Chromatography- Mass Spectrometry analysis

The aqueous extract of *Cyperus rotundus* was analysed for phytochemicals by GC-MS. The concentrated extract was dissolved in methanol, filtered, and injected (1µL) into the GC-MS system equipped with a capillary column. Helium was used as the carrier gas at a flow rate of 1mL/min. The oven temperature was programmed for optimal separation, and the compounds were identified by comparing their mass spectra with NIST library references (10).

Immunomodulatory activity of *Cyperus rotundus* by flow-cytometry on human PBMC

To study the immunomodulatory activity of *Cyperus rotundus* on human monocytes and antigen-presenting cells (APCs), PBMCs from healthy volunteers were isolated by density gradient centrifugation and cultured in RPMI-1640 medium. For the assay, the aqueous extract of *Cyperus rotundus* was applied at a concentration of 100µg/mL to the cultures for 6h. PBMCs, including both untreated and extract-treated groups, were incubated with or without standard activating agents (such as LPS) as positive controls.

After treatment, the cells were stained with fluorochrome-labelled monoclonal antibodies targeting the activation markers CD80 and CD86, together with lineage-specific markers (for example, CD14 for monocytes, CD11c for moDCs, and CD123 for pDCs). Flow cytometry was performed to quantify the surface expression of CD80 and CD86. An increase in the mean fluorescence intensity (MFI) of these markers indicated cellular activation in response to the extract. Data were analysed by comparing the MFI values of the treated and control groups. This flow cytometry-based approach reliably assesses the immunostimulatory potential of *Cyperus rotundus* extract on monocytes and dendritic cells through the upregulation of critical co-stimulatory molecules (11).

To evaluate the immunostimulatory activity of the *Cyperus rotundus* aqueous extract of *C. rotundus*, intracellular cytokine staining (ICS) was performed to measure Interleukin-6 (IL-6) and Tumor Necrosis Factor-alpha (TNF-α) levels in human peripheral blood mononuclear cells. PBMCs were isolated from healthy volunteers using density gradient centrifugation and cultured in RPMI-1640 medium at 1×10^6 cells/mL. Cells were treated with 100µg/mL of *C. rotundus* aqueous extract along with the positive controls, phorbol 12-myristate 13-acetate (PMA, 10ng/mL) and ionomycin (1µg/mL), to robustly stimulate cytokine production. Brefeldin A (10µg/mL) was added to inhibit protein transport, allowing the accumulation of intracellular cytokines during a 4-hour incubation at 37 °C with 5% CO_2.

After stimulation, the cells were surface-stained, fixed with 4% paraformaldehyde for 15 min, and permeabilised using a saponin-based buffer. Intracellular staining was performed using fluorochrome-conjugated monoclonal antibodies against IL-6 and TNF-α for 60 minutes. Flow cytometry was then used to analyse the stained cells, applying appropriate gating strategies to distinguish viable lymphocytes and quantifying cytokine-positive populations. Results were reported as the percentage of IL-6- and TNF-α-positive cells and mean fluorescence intensity, reflecting the immunomodulatory effect of the extract on cytokine production in PBMCs (12).

RESULTS

Cyperus rotundus contains various classes of phytochemicals responsible for its role in traditional medicine. Table 1 describes the classes of phytoconstituents reported to be present in the various extracts of *Cyperus rotundus*. All extracts of *Cyperus rotundus* contained phenolics, saponins, glycosides, steroids, terpenoids, flavonoids, and carbohydrates, which are major classes of phytochemicals.

In our study, Hep2cells were treated with aqueous, ethanolic, and dichloromethane extracts of *Cyperus rotundus* at concentrations ranging from 62.5 µg/mL to 1000 µg/mL for 24 h to assess the effectiveness of each extract on cell viability (Fig. 1). The results indicated that the aqueous extract at a concentration of 1000 µg/mL resulted in a cell viability of 80.1% at 24 h post-treatment. Additionally, when these cells were treated with the ethanolic extract at the same concentration, the viability was 77.8% at 24 h, and dichloromethane at 1000 µg/mL resulted in a cell viability of 43.32% (Table 2).

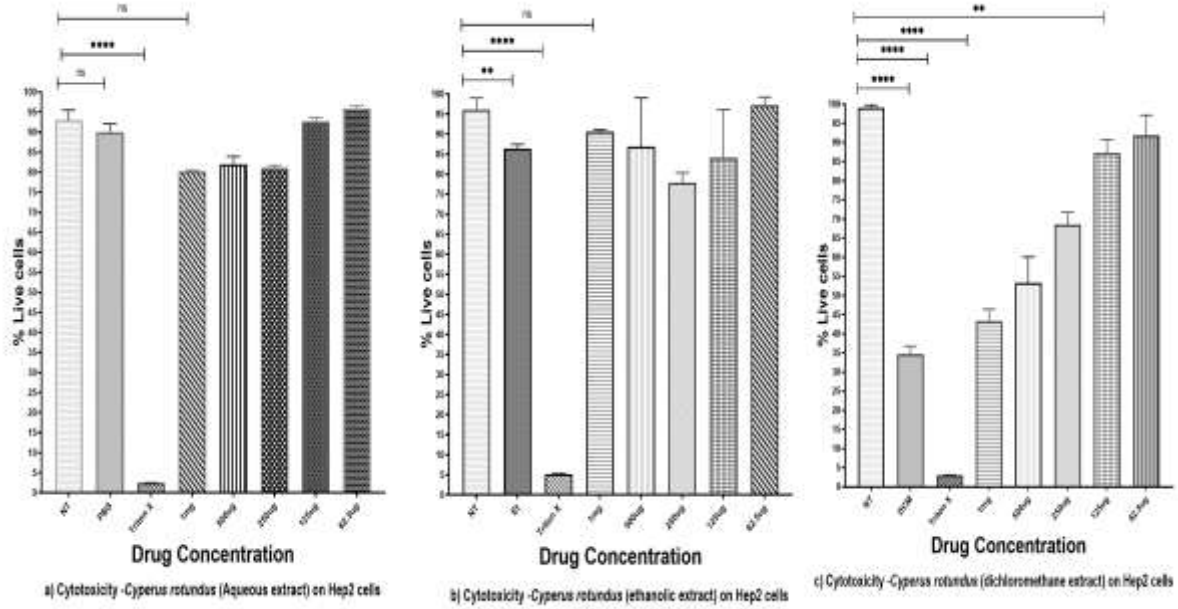


Fig. 1. In vitro MTT assay of Hep2 cells. Hep2 cells were incubated with different concentrations (1000,500,250,125 & 62.5 µg/mL) of aqueous, ethanol, and dichloromethane extracts of *Cyperus rotundus*. Growth inhibition was determined using the MTT assay. a) Aqueous, b) ethanol, and c) dichloromethane

Table1. Various classes of phytochemicals present in various extracts of *Cyperus rotundus*

Compound class	Test	*Cyperus rotundus*		
		Aqueous	Ethanol	Dichloromethane
Flavonoids	Alkaline reagent test	+	+	+
Tannins	Ferric Chloride Test	++	+	++
Carbohydrates	Molisch's test	+	+	+
Reducing sugars	Benedict's Test	+++	++	+++
Phenolics	Ferric chloride test	+	++	+
Glycosides	Borntrager's Test	+++	++	+++
Saponin	Foam test	-	+	+++
Steroids	Liebermann-Burchard's test	-	-	++
Terpenoids	Liebermann-Burchard's test	+	-	-

Dose-dependence of cytotoxicity

We investigated the effects of varying concentrations of the extracts on cytotoxicity. Hep2 cells were treated with 62.5ug/mL, 125ug/mL, 250ug/mL, 500ug/mL and 1 mg/mL extract for 24 h. These extracts caused cytotoxicity in different ways. The aqueous, ethanol and Dichloromethane extracts from *Cyperus rotundus* displayed cytotoxicity with IC50 values of 189.8 ± 1.57, 372.5 ± 2.04 and 263.7±5.26 ug/mL, respectively, against the Hep2 cell line (Fig. 2)

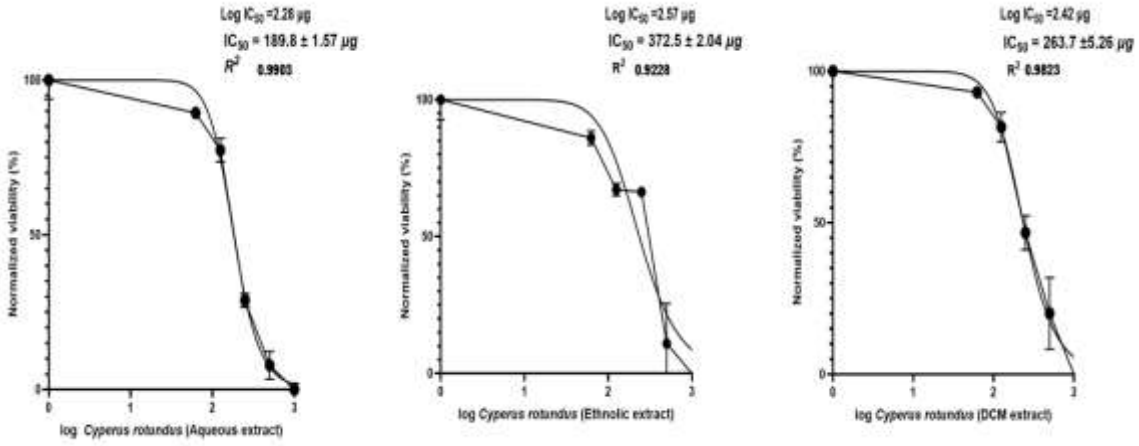
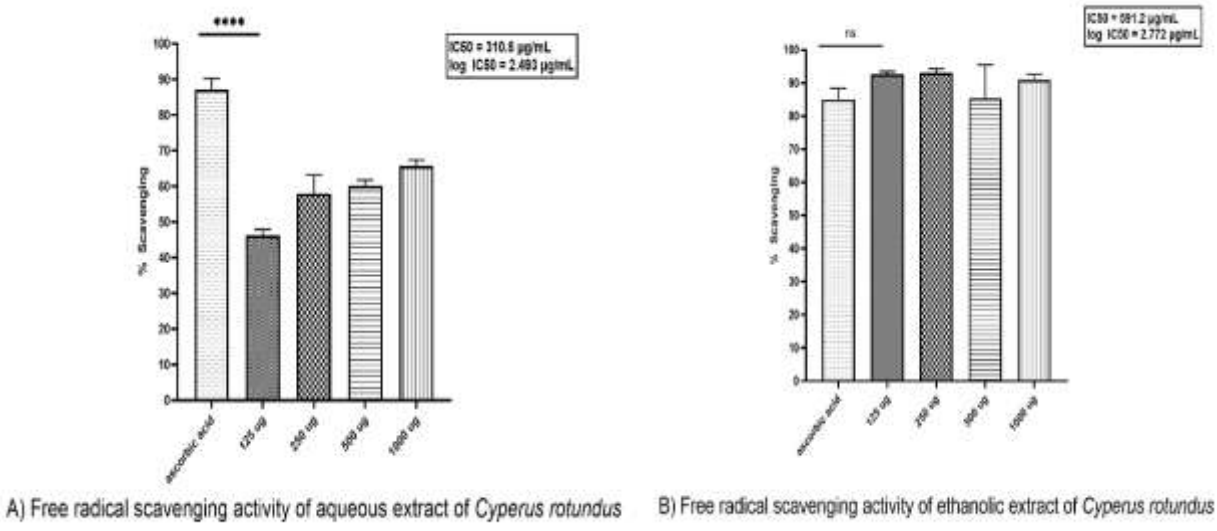


Fig 2 Dose-dependence of cytotoxicity - IC50 values were calculated by the mean percentage of cell death at different concentrations left to right: Aqueous, Ethanol and dichloromethane extracts.

DPPH Radical Scavenging Activity

An in vitro antioxidant assay of the aqueous and ethanolic extracts of *Cyperus rotundus* revealed their antioxidant potential. Percentage inhibition was observed through the scavenging of free radicals by the plant extract in a concentration-dependent manner. The antioxidant activity depends on the presence of total polyphenolic compounds. The DPPH radical scavenging activities of aqueous and ethanol extracts of *Cyperus rotundus* are shown in Fig. 3. Aqueous extracts of *Cyperus rotundus* had the DPPH radical scavenging activity, with an IC50 value of 310.8±2.50 µg/mL, and ethanolic extracts of *Cyperus rotundus* had an IC50 value of 591.2±0.81 µg/mL.



A) Free radical scavenging activity of aqueous extract of *Cyperus rotundus* B) Free radical scavenging activity of ethanolic extract of *Cyperus rotundus*

Fig. 3 Antioxidant activities of ethanolic and aqueous extracts of *Cyperus rotundus*. IC50 values were calculated by mean percentage of scavenging at different concentrations (125, 250, 500, and 1000 µg/mL) by DPPH assay.

Table 2

S.No	Concentration (µg/mL)	% Cell viability		
		CRE	CRD	CRA
1.	62.5	97.20±1.01	91.78±3.09	95.9±0.75
2.	125	90.53±0.46	87.14±1.79	92.6±0.95
3.	250	86.84±7.01	68.54±1.87	81.01±0.68
4.	500	84.11±6.90	53.31±3.90	81.92±1.94
5.	1000	77.78±1.27	43.32±1.55	80.11± 0.25
6.	IC_{50}	**372.5±2.04**	**263.7±5.26**	**189.8 ± 1.57**

Results were expressed as Mean ± SEM (n = 3) and analysed using one-way ANOVA followed by Tukey's post hoc test of significance. Statistical comparison between the mean % viability of each extract and its negative control was performed using one-way ANOVA followed by Dunnett's post hoc test of significance, wherein *p < 0.05 and **p < 0.01 were considered to be statistically significant and very statistically significant compared to the negative control. *C.rotundus* extracts were tested at concentrations of 1000,500,250,125 & 62.5 µg/mL

GC-MS analysis of the Aqueous extract of *Cyperus rotundus*

GC-MS analysis revealed that the aqueous extract of *Cyperus rotundus* contains forty-three distinct compounds. (Fig. 4 and Table 3).

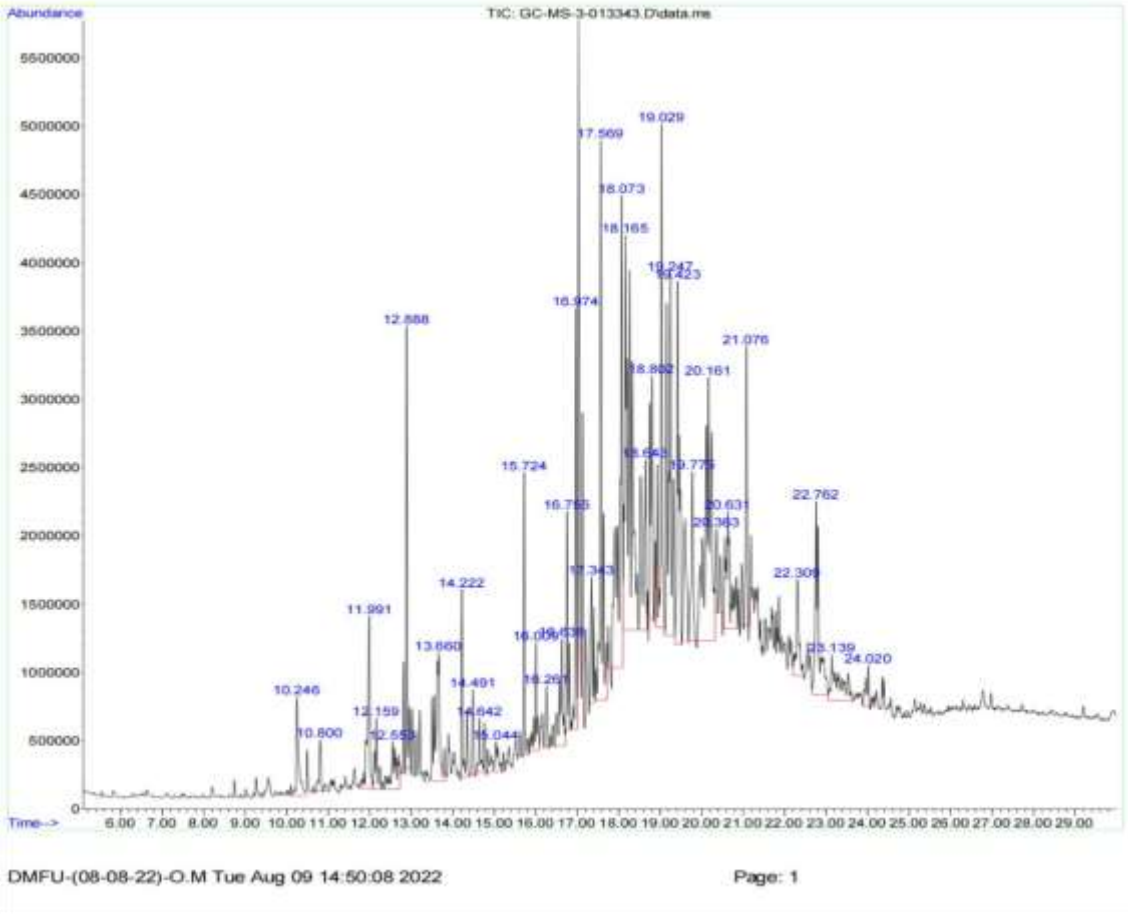


Fig. 4. GC-MS Chromatogram of aqueous extract of *Cyperus rotundus*

Table 3 shows list of compounds identified in the aqueous extract of *Cyperus rotundus* by GC-MS along with their CAS number and retention time

Peak #	Name	CAS	R time
1	Catechol	000120-80-9	10.246
2	cyclopentadiene 2 5 5-trimethyl-	007086-15-9	10.8
3	4,4-Dimethylcyclohexadienone	001073-14-9	11.991
4	Cyclohexanol, 3,5-dimethyl	005441-52-1	12.159
5	Cyclohexanone, 3-ethenyl	001740-63-2	12.553
6	2-Hydrazinopyridine	004930-98-7	12.888
7	1,4-Benzenediol, 2,6-dimethyl	000654-42-2	13.66
8	Myrtenol	000515-00-4	14.222
9	2-cyclobutylpropan-2-ol	059383-67-4	14.491
10	2,4-Hexadiene, 2,5-dimethyl	000764-13-6	14.642
11	3-Methyl-1,4-pentadien-3-ol	000918-86-5	15.044
12	Diethyl Phthalate	84-66-2	15.724
13	DIHYDROMYRCENE	2436-90-0	16.009
14	1h-1,3a-ethanopentalen-4-ol, Hexahydro-, Cis-	117221-75-7	16.261
15	Cyclopropanecarboxylic acid, 2,2-dimethyl-3-(2-propenyl)-	074685-76-0	16.638
16	cis-p-Mentha-1(7),8-dien-2-ol	1000374-16	17.343
17	5-Methyl-3-octyne	062108-33-2	17.569
18	Tridecanedial	063521-76-6	17.569
19	2-methyl-3-methylidenecyclopentane-1-carbaldehyde	826337-64-8	18.073
20	Farnesol	4602-84-0	18.165
21	cis-Z-.alpha.-Bisabolene epoxide	1000131-71-2	18.643
22	4,8,13-Duvatriene-1,3-diol	7220-78-2	18.643
23	3-Isoxazolecarboxamide, 4,5-dihydro-5-methyl-5-(4-methyl-5-oxo-3-cyclohexenyl)-	1000362-31-0	18.802
24	1S,2S,5S,8S,9R)-4,4,8-trimethyltricyclo[6.3.1.01,5]dodecane-2,9-diol	1000191-00-6	19.029
25	Cyclohexane, 1,3-dimethyl-2-methylene-, trans	20348-74-7	19.247
26	2-Buten-1-ol, 3-methyl-, acetate	1191-16-8	19.423
27	Dihydrocarvyl acetate	20777-49-5	19.775
28	cis-vaccenic acid	693-72-1	20.161
29	cis-13-Octadecenoic acid	13126-39-1	20.161
30	3-Methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-ol	070172-00-8	20.363
31	trans-Geranylgeranio	24034-73-9	20.631
32	8-Hydroxycarvotanacetone	131486-99-2	21.076
33	4-Isopropenyl-4,7-dimethyl-1-oxaspiro[2.5]octane	1000187-81-7	22.309
34	Patchoulane	25491207	22.762
35	Alloaromadendrene oxide	85760-81-2	23.139
36	Bis(2-ethylhexyl) phthalate	117-81-7	24.02
37	Hydrazinium, 1,1,1-trimethyl-2-[[(phenylsulfonyl)amino]methylene]-, hydroxide	035468-56-5	14.491
38	2-Furanmethanol, tetrahydro-.alpha.,.alpha.,5-trimethyl-5-(4-methyl-3-cyclohexen-1-yl)-, [2S-[2.alpha.,5.beta.(R*)]]-	26184-88-3	15.044
39	DIETHYL PHTHALATE	000084-66-2	15.724
40	Cyclohexanol, 2-methyl-5-(1-methylethenyl)-, (1α,2α,5β)-	18675-33-7	16.974
41	1,6-Octadiene, 3,7-dimethyl-	2436-90-0	16.009
42	1H-1,3a-Ethanopentalen-4-ol, hexahydro-, cis	117221-75-7	16.261
43	1,2-Cyclohexanedimethanol	003971-29-7	16.756

To study the immunomodulatory activity of *Cyperus rotundus*, we stimulated PBMCs isolated from healthy volunteers with *Cyperus rotundus* aqueous extract at 100 µg/ml for 6 h. Flow cytometry was used to evaluate the activation potential

of the aqueous extract of *Cyperus rotundus* and fluorochrome-tagged antibodies were used to characterize monocytes. Fig. 5 shows the gating strategy employed to characterize monocytes, where monocytes are defined as CD3- CD20- CD14+. We used classical activation markers such as CD80 and CD86 to study the activation state of monocytes after treatment with the extract (Fig. 6). Treatment with aqueous extract enhanced the expression of monocyte activation markers (Fig. 6). We compared the expression of monocyte activation markers to that in untreated cells, denoted as US (Unstimulated). LPS (Sigma, USA) was used as a positive control. Treatment with the extract increased the expression of activation markers by thirteen-fold (CD80+CD86+).

Furthermore, we explored whether *Cyperus rotundus* extract could augment the expression of these co-stimulatory molecules on the surface of APCs, such as monocyte-derived dendritic cells and plasmacytoid dendritic cells.

MoDCs were characterised based on their expression of HLADR and CD11c. Fig. 5 shows the gating strategy employed to characterize this subset of DCs. Upon treatment with the aqueous extract of *Cyperus rotundus*, we observed increased expression of both co-stimulatory molecules - CD80 and CD86 (Fig. 6). Treatment with the extract increased the expression of CD80+CD86+ by eight-fold (Fig. 7).

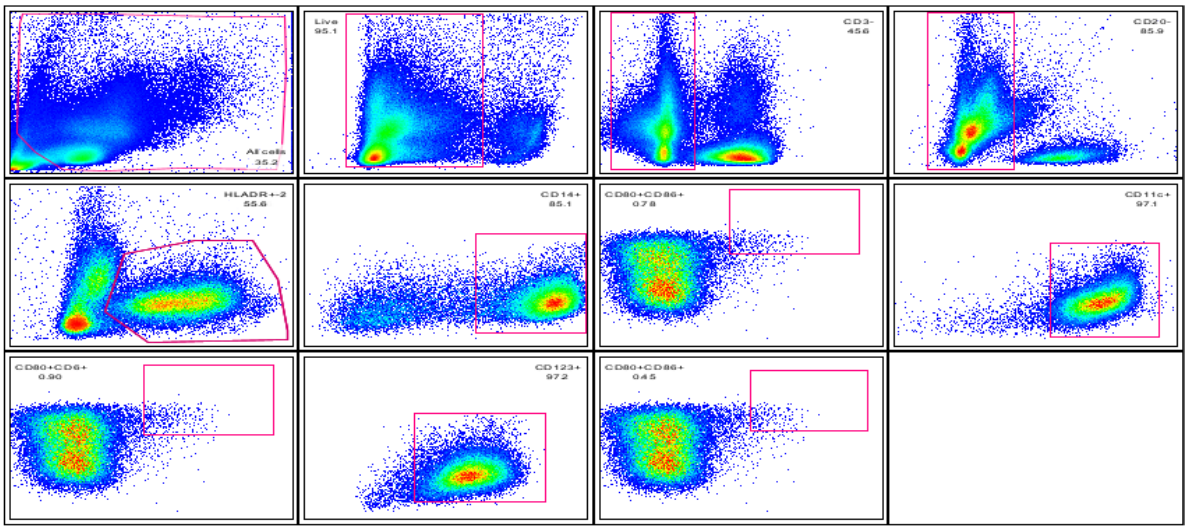


Fig. 5 shows the gating strategy employed to characterize monocytes, where monocytes are defined as CD3- CD20- CD14+.

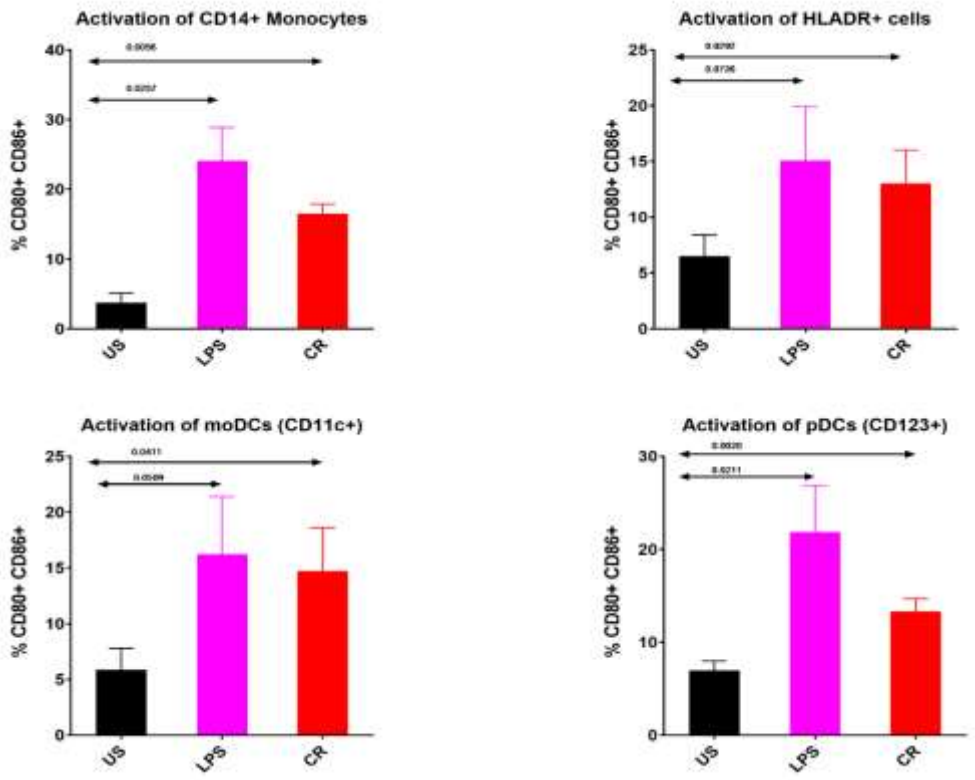


Fig 6 shows the enhanced expression of surface activation markers (CD80 and CD86) on monocytes, HLA-DR+ cells, monocyte-derived dendritic cells and plasmacytoid dendritic cells after treatment with aqueous extract of *Cyperus rotundus* (100 µg/mL). US – unstimulated. LPS – Positive control and CR - aqueous extract of *Cyperus rotundus*. $p < 0.05$

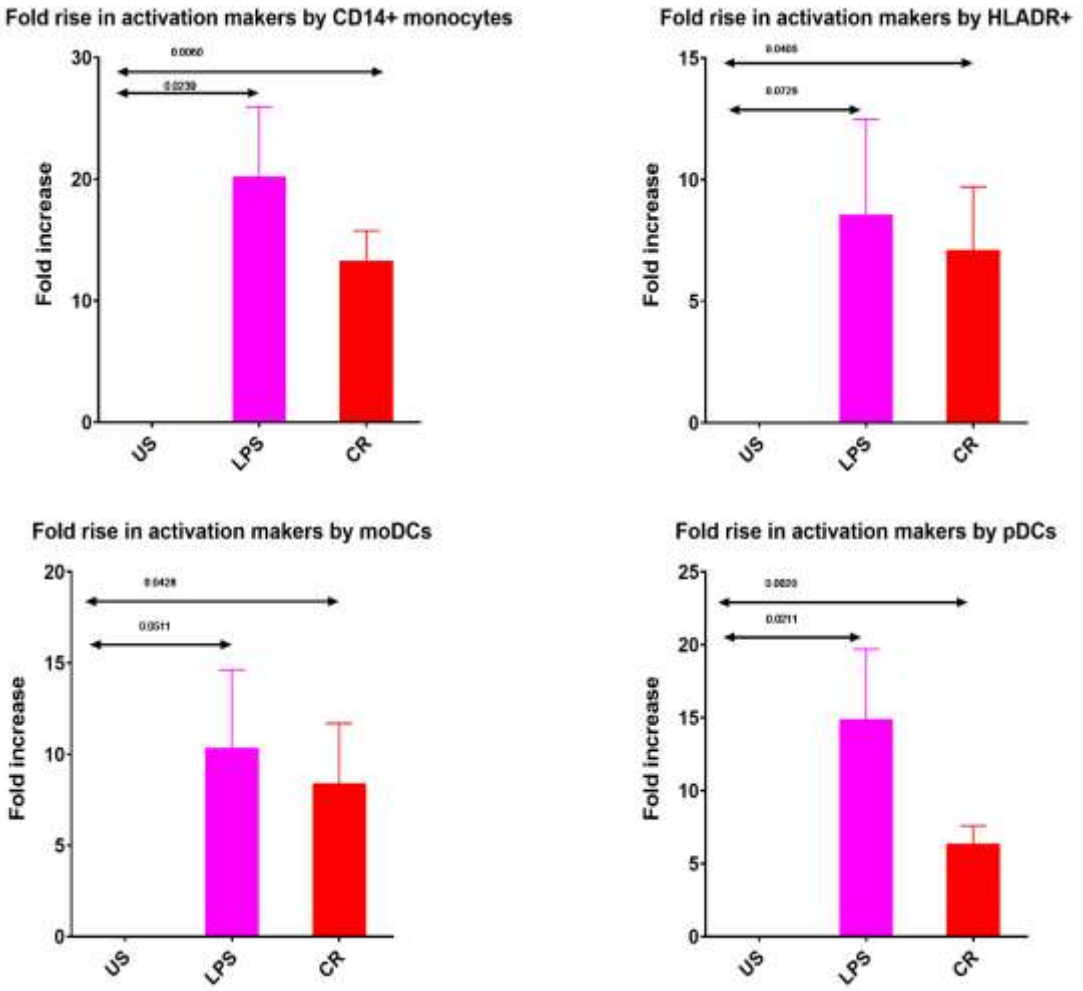


Fig 7 shows the fold increase in the expression of surface activation markers (CD80 and CD86) on monocytes, HLA-DR+ cells, monocyte-derived dendritic cells and plasmacytoid dendritic cells after treatment with aqueous extract of *Cyperus rotundus* (100 µg/mL) $p < 0.05$

Plasmacytoid dendritic cells (pDCs) are an essential subset of dendritic cells involved in antigen presentation. These cells were characterized by the expression of HLADR+ and CD123+. The pDCs are typically represented as CD3-CD20-CD14-HLADR+CD11c-CD123+ cells. In the next part of the study, we tested the aqueous extract of *Cyperus rotundus* for its ability to activate pDCs by evaluating its ability to enhance the expression of co-stimulatory molecules in these cells. After treatment with the extract, the pDCs showed a six-fold increase in CD80 + CD86 + expression (Fig.7).

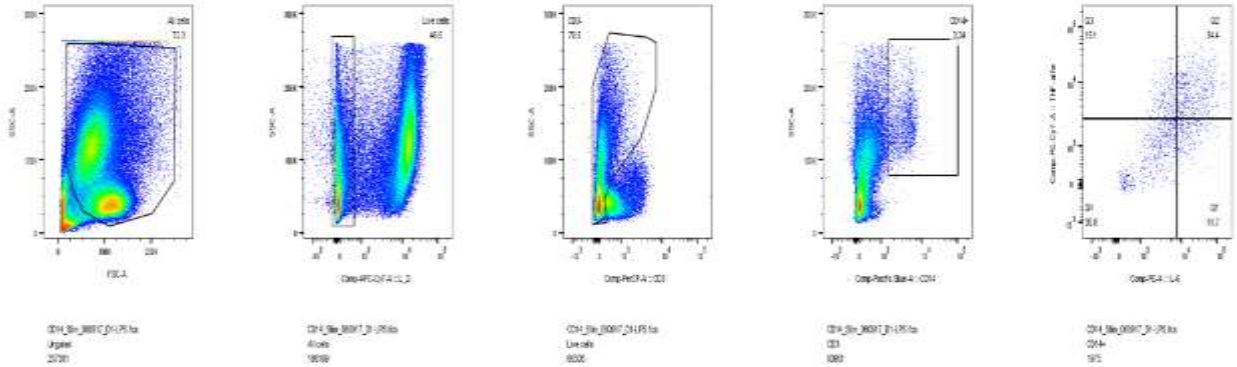


Fig. 8 shows the gating strategy employed to characterise intracellular expression of TNF-α and IL-6 in monocytes by ICS.

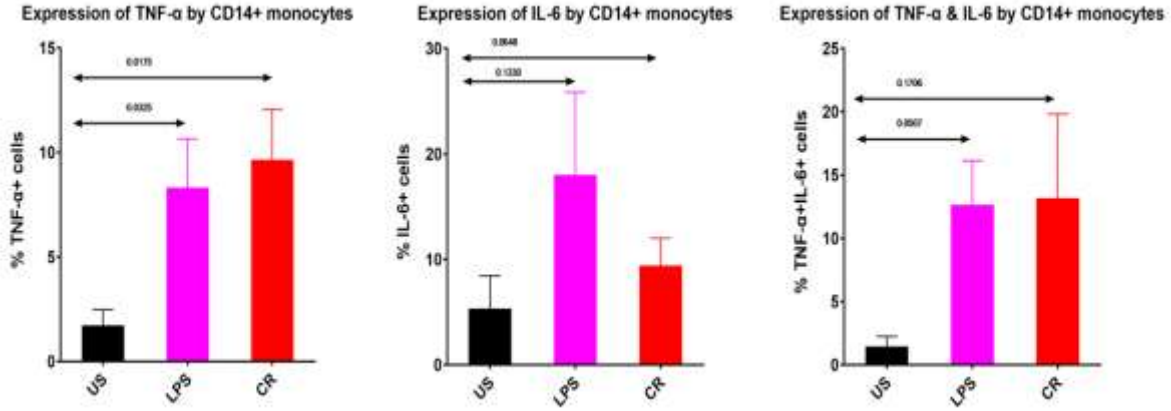


Fig. 9 shows the enhanced intracellular expression of cytokines TNF-α, IL-6 and both in monocytes by ICS after treatment with aqueous extract of *Cyperus rotundus* (100 µg/mL), US-unstimulated. LPS – Positive control and CR - aqueous extract of *Cyperus rotundus*. $p < 0.05$

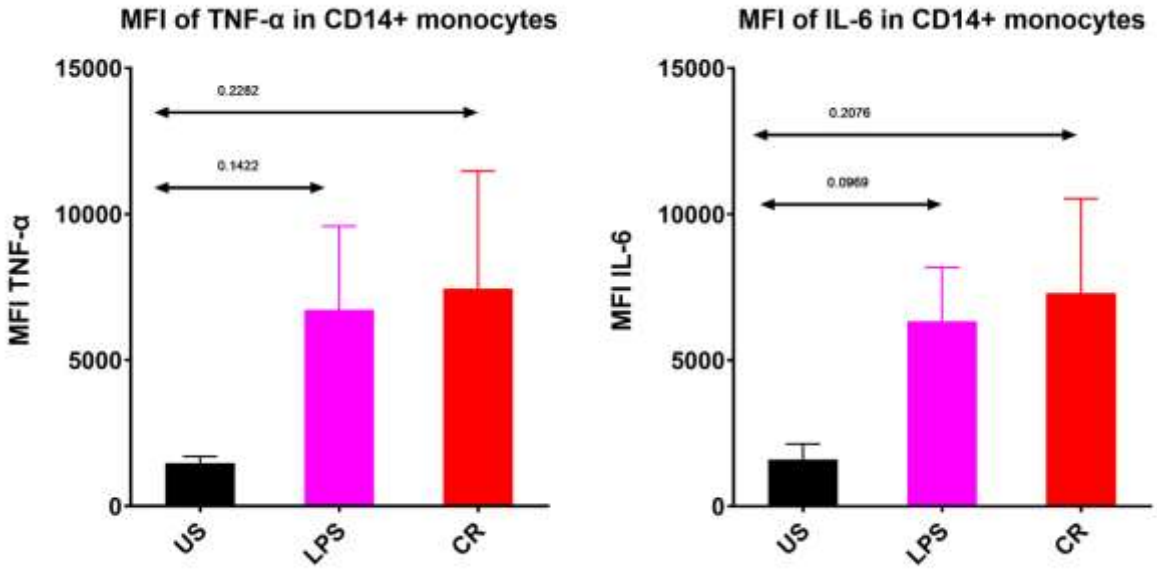


Fig. 10 shows the effect of aqueous extract of *Cyperus rotundus* on the Mean Fluorescence Intensity (MFI) of TNF-α and IL-6 in monocytes by ICS after treatment with aqueous extract of *Cyperus rotundus* (100 µg/mL), US-unstimulated. LPS – Positive control and CR - aqueous extract of *Cyperus rotundus*. $p < 0.05$

We further evaluated the immunostimulatory properties of the *Cyperus rotundus* aqueous extract. PBMCs were isolated from healthy volunteers and treated with aqueous extract at a concentration of 100 µg/ml for 4 h. Following stimulation, the cells were harvested and stained intracellularly for: Interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α). This Intracellular Cytokine Staining captures the unsecreted cytokines present inside the stimulated cells, which were studied by flow cytometry. Earlier experiments showed the effect of an aqueous extract of *Cyperus rotundus* on the expression of markers on the cell surface. For effective functioning of immune cells, it is not only necessary for them to be activated, but they also need to be functional, as evidenced by increased cytokine expression. The gating strategy employed for the ICS is shown in Fig.8. We observed that exposure of monocytes to the extract stimulated the expression of both IL-6 and TNF-α. We observed a nine-fold increase in TNF-α and Interleukin-6 expression, with a thirteen-fold increase in their combined expression (Fig. 9). There was a five-fold increase in the Mean Fluorescence Intensity (MFI) of both TNF-α and IL-6 (Fig. 10).

DISCUSSION

Traditional herbal medicines contain synergistic phytochemicals with proven efficacy and safety. Their acceptance stems from cultural traditions, fewer side effects, and affordability in resource-limited settings. Healthcare providers choose natural alternatives due to concerns over chronic diseases and conventional therapy side effects. WHO endorsement supports integrating complementary medicine with modern medicine. These systems address non-communicable diseases through lifestyle counselling and herbal formulations (13), shown to accelerate viral clearance in mild COVID-19 (14). Their therapeutic benefits and cost-effectiveness help their incorporation into standard healthcare practices. Immunomodulators enhance or suppress immune responses (3). Immunostimulants boost cytokine production and host

defense (15), while checkpoint inhibitors activate lymphocytes against tumors (16). Immunosuppressants prevent transplant rejection and control autoimmune diseases (17). Herbal extracts' immunomodulatory properties attract attention due to their bioactive compounds (18). They offer safer alternatives to conventional agents with cytotoxic effects (19). These remedies modulate cytokines and stimulate immune cells (20), with multi-target actions beneficial for immune dysregulation (18).

Cyperus rotundus is a perennial sedge 30-70 cm tall, with triangular stems from rhizomes-bearing tubers. Leaves are 10-60 cm long, with umbels of reddish-brown spikelets and triangular achenes 1-1.5 mm long. (5). Plant rhizomes contain bioactive compounds with therapeutic potential (21, 22). Traditional medicine uses it for treatment of digestive disorders, inflammation. It has been proved for its analgesic and neuroprotective effects (23). Also, previous studies shows that *Cyperus rotundus* has anti-diabetic properties and anticancer activity. It is being used to treat spasms and menstrual irregularities (21). Earlier research validates its antimicrobial effects through bioactive compounds (4).

The phytochemical profile of *Cyperus rotundus* contains diverse secondary metabolites that contribute to its biological activities. These include essential oils, flavonoids, alkaloids, and terpenoids, along with α-cyperone, β-selinene, cyperol, and cyperene being the major oil components. (21). Our study showed that the aqueous extract contained glycosides, reducing sugars, tannins, flavonoids, phenolics, and terpenoids, whereas the ethanolic extract contained glycosides, reducing sugars, phenolics, saponins, tannins, carbohydrates, and flavonoids. The dichloromethane extract contained glycosides, saponins, reducing sugars, and steroids but lacked terpenoids.

Studies have shown that *Cyperus rotundus* extracts exhibit solvent-dependent cytotoxicity. The aqueous extracts displayed minimal toxicity, maintaining over 80% viability in cancer cell lines at 200 µg/mL, while our study showed an IC50 of 190 µg/mL in Hep2 cells. Studies show ethanolic extracts demonstrate IC_{50} between 50-150 µg/mL in MCF-7, HeLa, and other tumour models, whereas we found an IC50 of 372 µg/mL in Hep2 cells. Dichloromethane (DCM) fractions the exhibited most potent cytotoxicity, with IC_{50} values ranging from 25-75 µg/ml(24). In contrast, our study revealed an IC_{50} of 264 µg/ml. Further bioassay-guided fractionation and mechanistic research are needed to isolate cytotoxic compounds and understand their apoptotic pathways.

Earlier studies of aqueous and ethanolic extracts of *Cyperus rotundus* showed concentration-dependent antioxidant activity, with IC_{50} values of 337µg/mL for aqueous extract (25)and 447µg/mL for ethanolic extract(26). These results are consistent with the high polyphenol and flavonoid content of both extracts, which are responsible for their antioxidant effects. Our study found that the aqueous extract had DPPH radical scavenging activity, with an IC50 of 310 µg/mL, whereas the ethanolic extract showed an IC50 of 590µg/mL. These findings support the traditional use of C. rotundus as a natural antioxidant and warrant further research on oxidative stress-related disorders.

Previous gas chromatography-mass spectrometry (GC-MS) analyses of aqueous extracts of *Cyperus rotundus* have revealed diverse bioactive phytochemicals, supporting its traditional medicinal uses (27). GC-MS analysis of the aqueous rhizome extract revealed several phenolic compounds with antioxidant and enzyme-inhibitory properties(28). Our GC-MS results identified forty-three compounds with established free radical scavenging or enzyme inhibitory actions. GC-MS profiling confirmed that the aqueous extract of C. rotundus contained phenolic and aromatic constituents, which may contribute to its therapeutic use as a source of phytomedicines(29).

Previous studies have shown the immunomodulatory potential of *Cyperus rotundus* extracts, which is attributed to flavonoids, terpenoids, and phenolic compounds(30). In vitro experiments have demonstrated that aqueous and ethanolic extracts enhance lymphocyte proliferation (31). However, this is the first study to evaluate the immunomodulatory activity of *Cyperus rotundus* aqueous extract on innate immune cells. The study showed that the extract upregulated monocyte and dendritic cell activation, particularly MoDCs and PDCs, through increased expression of co-stimulatory molecules (CD80 and CD86). Intracellular Cytokine Staining showed that the extract enhanced IL-6 and TNF-α secretion, strengthening host defense against pathogens.

Previous in vivo studies corroborated our findings, showing that *Cyperus rotundus* extracts enhance humoral and cell-mediated immune responses (32). The immunostimulatory and anti-inflammatory properties of C. rotundus suggest its adaptogenic role in balancing the immune function. These findings support its traditional medicinal use as an immunomodulator and suggest its potential in the treatment of immune-related conditions.

CONCLUSION

This study highlights the growing importance of traditional herbal medicines, especially in resource-limited settings, owing to their affordability, safety, and multitarget efficacy. Driven by the limitations of conventional drugs, the integration of systems such as Ayurveda and Siddha can help address chronic and infectious diseases by leveraging holistic regimens and phytochemical-rich formulations. *Cyperus rotundus*, a widely used medicinal plant, has a complex phytochemical profile with proven antioxidant, cytotoxic, and immunomodulatory activities. Our study confirmed its solvent-specific cytotoxicity, substantial DPPH scavenging, and diverse array of bioactive compounds, notably phenolics and flavonoids, as revealed by the GC-MS analysis. Most significantly, we demonstrated for the first time that the aqueous extract of C. rotundus robustly activates innate immune cells by upregulating key co-stimulatory markers and inducing cytokines IL-6 and TNF-α. These findings not only validate the plant's ethnomedicinal reputation as an immunomodulator but also indicate its potential as a safe phytomedicine for managing oxidative stress and immune dysfunction.

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Availability of data and materials
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Consent for publication
Yes, we provide our consent for the publication of this research article.

Competing interest
The authors declare that they have no competing interests

Ethical Compliance: All procedures performed in studies involving human participants were with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of Interest declaration: The authors declare that they have no affiliations with or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

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