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Phytochemical Profiling and Anti-Inflammatory Activity of Leaf Extract from *Cleome gynandra* Linn. and *Melicope ptelefolia* Champ. ex Benth

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Herbal remedies are increasingly studied for their potential as safer alternatives to conventional anti-inflammatory drugs. However, the anti-inflammatory efficacy and interaction of many herbal combinations remain poorly understood. This study addresses this gap by investigating the anti-inflammatory activity, herb-herb interactions, and LC-ESI-MS/MS metabolite profiles of Cleome gynandra and Melicope ptelefolia leaf extracts. Fresh leaves of both plants were extracted and tested for cytotoxicity and nitric oxide (NO) inhibition using the murine RAW 264.7 macrophage cell line. Cell viability was assessed via MTT assay, while NO inhibition was measured for individual and combined extracts at varying ratios. LC-ESI-MS/MS analysis was performed to identify the major bioactive metabolites. Cleome gynandra extract showed higher cell viability compared to M. ptelefolia, indicating lower cytotoxicity. Among all tested combinations, the 70:30 (w/w) ratio of C. gynandra to M. ptelefolia exhibited the highest cell viability. Although C. gynandra demonstrated limited NO inhibition at 0.5 mg/mL, it still more potent than M. ptelefolia and their formulations. The LC-MS/MS analysis revealed that M. ptelefolia contains flavonoids like kaempferol neohesperidoside and derivatives with sugars such as rhamnose and arabinose, known to inhibit nitric oxide synthase and reduce NO production. Isorhamnetin derivatives also support its anti-inflammatory potential by modulating signaling pathways. In C. gynandra, the presence of quercetin rutinoside (rutin), kaempferol rutinoside, and phenolic acids like caffeic acid contribute to its anti-inflammatory effects. Quercetin rutinoside inhibits enzymes like COX-2 and iNOS and suppresses cytokines such as TNF-a and IL-6. Caffeic acid, a potent antioxidant, inhibits NF-?B activation, collectively supporting its anti-inflammatory capacity. In conclusion, C. gynandra demonstrates promising potential as a safer anti-inflammatory agent compared to M. ptelefolia, although its efficacy remains lower than that of conventional drugs such as diclofenac sodium.

Keywords: Anti-inflammatory; Cleome gynandra; Liquid Chromatography - Mass Spectrometry (LCMS); Melicope ptelefolia; Nitric Oxide (NO) inhibition; Cytotoxicity; Herb-herb Interactions.

Cleome gynandra (Cleomaceae), commonly known as 'Maman,' is a widely consumed leafy vegetable and a well-known herb in traditional Indian medicine, used for treating various inflammatory conditions.[1] Traditionally, its leaves and seeds have been used to manage ailments such as ear inflammation, epileptic seizures, stomachaches, constipation, and other inflammation-related disorders.[2] It is particularly popular among low-income communities due to its affordability and high nutritional value, being rich in vitamins A and C, as well as essential minerals like iron and calcium.[3-5] Extensive research has demonstrated the biological activities of *C. gynandra*, including antidiabetic,[6] antioxidant,[7] anti-inflammatory,[4] and anticancer effects.[8] Phytochemical analysis has also confirmed the presence of bioactive compounds such as flavonoids, tannins, alkaloids, terpenoids, steroids, and carotenoids, which contribute to its pharmacological potential.[9]

Melicope ptelefolia (Rutaceae), locally known as 'Tenggek burung' in Malaysia,[10] 'Uam sam ngam' in Thailand, and 'Sampang uam' in Indonesia, is another herb traditionally used in Southeast Asia.[11] In Malaysia, the young leaves and shoots are commonly consumed fresh as 'ulam' (traditional salad)[12] and are believed to possess medicinal properties, particularly for reducing inflammation and treating skin irritations.[13] Scientific studies have reported various pharmacological properties of *M. ptelefolia* leaf extract, including antipyretic, antioxidant, analgesic, and anti-inflammatory effects. These benefits are largely attributed to its rich content of benzopyrans, alkaloids, and glycosides.[14-18]

Inflammation, although a natural part of the healing process, is often associated with pathological consequences when prolonged or uncontrolled.[19] Macrophages play a central role in initiating and regulating inflammation by releasing nitric oxide (NO), pro-inflammatory cytokines, and other mediators in response to stimuli such as bacterial lipopolysaccharides (LPS).[20] While non-steroidal anti-inflammatory drugs (NSAIDs) like diclofenac, aspirin, and indomethacin are commonly used to manage inflammation,[21-22] their long-term use is associated with serious side effects, including gastrointestinal bleeding, renal toxicity, hypertension, and cardiovascular risks.[23-25]

Given the drawbacks of NSAIDs, there is increasing interest in exploring herbal alternatives that are believed to be safer and less toxic.[26] Both *C. gynandra* and *M. ptelefolia* have demonstrated anti-inflammatory potential individually. However, a significant gap in the literature exists regarding the combined use (herb-herb interaction) of these two plants as a formulation for anti-inflammatory therapy. Most studies to date have focused on single-herb activity,[27] with little understanding of possible synergistic or antagonistic effects when used in combination.[28]

To address this research gap, this study aims to evaluate the cytotoxicity and anti-inflammatory properties of *C. gynandra*, *M. ptelefolia*, and their various formulations. In addition, LC-ESI-MS/MS-based metabolite profiling will be conducted to identify the active compounds responsible for their pharmacological activities.[29] By optimizing the extraction protocol and analyzing both individual and combined effects, this study seeks to contribute to the development of effective and safer herbal-based anti-inflammatory treatments.

MATERIALS AND METHODS

This study did not involve human participants, animals and therefore, ethical approval or informed consent was not required.

Chemicals and Reagents

Analytical-grade chemicals and solvents were all utilized. Saline phosphate buffer, 3-[4,5-dimethylthiazol-2-yl]Sigma-Aldrich Co. (St. Louis, MO, USA) supplied the 2,5-diphenyltetrazolium bromide (MTT), diclofenac sodium salt, Griess reagent, and lipopolysaccharides (LPS); SciencellTM (San Diego, CA) supplied trypan blue; HiMedia® Laboratories (Mumbai, India) supplied Dulbecco's Modified Eagle Medium (DMEM); Amresco Inc. (Ohio, USA) supplied sodium dodecyl sulfate and dimethyl sulfoxide (DMSO); Fisher Scientific (Loughborough, Leicestershire, UK) supplied fetal bovine serum (FBS); and Gibco (Life Technologies, USA) supplied the antibiotic mixture of penicillin-streptomycin.

Collection of plant Material

Fresh *C. gynandra* and *M. ptelefolia* leaves were supplied by the Biotechnology and

Nanotechnology Research Centre, Malaysian Agricultural Research and Development Institute (MARDI), Malaysia. The leaves were collected before noon to maintain their freshness. A voucher specimen of *C. gynandra* (voucher specimen number MDI 12840) and *M. ptelefolia* (voucher specimen number MDI 12450) were deposited in MARDI's herbarium. The plant was verified by a botanist, Dr. Mohd Norfaizal Ghazalli. Lab tissue paper was used to gently dry the gathered leaves after they had been completely cleaned with tap water. A mortar and pestle were then used to grind the clean leaves into a powder in liquid nitrogen. Prior to examination, the powder samples were held at -20 °C after being instantly freeze-dried using a Labconco 6 Plus freeze drier in Kansas City, USA.

Extraction of Samples

The extraction of the powder leaves was performed as described by Chandradevan et al.[30] with slight modification. Generally, 0.5 g of grounded and dried powder of leaves of *C. gynandra* and *M. ptelefolia* were macerated individually in 70% methanol at a ratio of 1:80 (w/v). The grounded and dried powder leaves of *C. gynandra* and *M. ptelefolia* were extracted individually in 40 ml of 70% methanol, respectively. The mixture was then homogenised for 1 min at 262 *g* using a homogeniser (Ultra Turrax, IKA, Germany), followed by shaking at room temperature for 30 min at 72 *g* using an orbit shaker (Multi Reax, Heidolph, Germany). A 125 mm diameter filter paper (Advantec, Japan) was used to filter the supernatant. A rotary evaporator (RII, Buchi, Switzerland) was used to concentrate the collected supernatant to create a crystallized extract.

Herbal Combinations

Herbal combinations were prepared by mixing the crystallised *C. gynandra* and *M. ptelefolia* extract in a ratio of 30:70 (w/w), 50:50 (w/w) and 70:30 (w/w) based on previously reported herbal formulation study.[31]

Cell Culture

The American Type Culture Collection (ATCC®) (ATCC accession no. TIB-71) (Manassas, VA, USA) provided the murine RAW 264.7 macrophage cell line. The cells were cultivated in DMEM with 5% FBS added, and they were then incubated at 37 °C in a humidified room with 5% CO2. Each experiment employed cells with less than 18 passage numbers.

MTT Assay

The MTT assay of the *C. gynandra* and *M. ptelefolia* and its herbal formulation extracts in RAW 264.7 cells were performed as described by Chung et al.[32] with slight modifications. Concisely, the cells (2×10^4 cells per well) were seeded in a 96-well plate and incubated for 24 h Different concentrations of the individual herbs and herbal formulation (0.25–10 mg/mL) were applied to the adhering cells. After 24 hours of treatment, 30 µL of the MTT solution (2 mg/mL) was applied to each well, and the wells were then incubated for 4 hours at 37°C in a dark environment. To dissolve the formazan crystals, 100 µL of DMSO was applied to each well after the supernatant was aspirated. A microplate reader (Biotek EL800, Cambridge Scientific Products, Watertown, USA) set to 570 nm was used to measure the absorbance. The following formula was used to calculate the percentage of cell viability. LPS-induced RAW 264.7 was then used to further select the non-cytotoxic concentration with at least 90% cell viability for the anti-inflammatory test. The MTT assay for each of the extract was conducted in triplicate of three independent experiments. Detailed calculation of cell viability is published previously.[32]

Nitric Oxide (NO) Inhibition Activity

To determine the anti-inflammatory activity of *C. gynandra*, *M. ptelefolia* extract and its herbal formulation, the NO inhibition assay was performed according to procedure described by Chandradevan et al.[26] with slight modification. In brief, RAW 264.7 cells (5×10^4 cells per well) were seeded in 96-well plates and left in an incubator for overnight. Then, cells were induced with 10 µg/mL LPS (50 iL, diluted in culture media), followed by treatment with different concentration of plant extract (0.25–10 mg/mL, w/v). Diclofenac sodium salt (0.6 µM) was used as the positive control. The treated cells were incubated in an environment containing 5% CO_2 for 24 hours at 37°C. Griess reagent was used to determine the studied extracts' inhibitory impact on NO generation. Following a 24-hour incubation period, 50 µL of the plate's medium was cautiously moved into a fresh 96-well plate. After adding the same amount of Griess

reagent to each well, the plate was placed in the dark for ten minutes. Sodium nitrite ($NaNO_2$) at varying concentrations (0.156 to 100 µM) served as a standard control in this experiment. A microplate reader (E16 OneTech Medical Equipment Co., Ltd., Guangzhou, China) was then used to measure the absorbance at 540 nm. The NO inhibition assay for each tested extract was performed in triplicate of three independent experiments. Detailed calculation is published previously.[26]

Separation and tentative identification of selected peaks via LC-ESI-MS/MS

Separation and identification of metabolites were achieved according to the method previously described in Chandradevan and Indu Bala.[33] A HPLC system (1200 Series, Agilent Technologies, Germany) was paired with a mass spectrometry (3200 QTrap, ABSciex, USA) was used in this study. A reversed phase C18 column (Thermo Hypersil, 5 µm, 150 x 4.6 mm, Thermo Scientific, USA) was used to ease the separation. Ten microliter of extracts from both herbs at 20 mg/mL was injected into HPLC with the flow rate set at 1 mL/min. A gradient setting was set using 0.1% formic acid as solvent A and 0.1% formic acid in acetonitrile as solvent B. The gradient setting was set as follow: Solvent B was increased from 5% to 40% in 40 min before further increased to 95% for 15 min. A steep decrement of solvent B to 5% within a minute and maintained at the same percentage for another 5 min for equilibrating the column. Ionisation of metabolites was done using electron spray ionization (ESI) at negative mode. The ion source was heated up at 500°C and the entrance potential, de-clustering potential and collision energy were set at -15V, -20V and -25V respectively. Enhance mode was activated in order to obtain the MS/MS fragmentations. The entire analysis was performed using Analyst software (version 1.4.2). Identification of selected peaks from generated chromatograms was done by comparing their mass fragments with previously reported data and online databases. [34, 35]

Statistical Analysis

The mean ± standard error of mean (SEM) was used to represent the data. The Statistical Program for Social Sciences (SPSS for Windows, Version 22) (International Business Machines Corp. IBM, New York, USA) was used to do the statistical analysis, and a p-value of less than 0.05 was deemed significant. One-way analysis of variance (ANOVA) was used to evaluate the results, and the Tukey or Dunnet tests were used to determine whether the plant extracts differed significantly.

RESULTS

Cytotoxicity of Raw 264.7 Cells Treated With *C. Gynandra, M. Ptelefolia*, and Herbal Formulations

Following a 24-hour treatment, the cell viability percentages of RAW 264.7 macrophages significantly decreased ($p < 0.05$) in a concentration-dependent manner upon exposure to various concentrations (0.25–10 mg/mL, w/v) of the single herbs *C. gynandra* and *M. ptelefolia*. Specifically, *C. gynandra* (Figure 1A) demonstrated a higher tolerated concentration, with cells maintaining at least 90% viability at doses below 4 mg/mL, whereas *M. ptelefolia* achieved similar viability only at concentrations below 1 mg/mL. Notably, *C. gynandra* exhibited the highest percentage of cell viability compared to *M. ptelefolia*, indicating that *C. gynandra* is less cytotoxic.

When evaluating the herbal formulations combinations of *C. gynandra* and *M. ptelefolia* at ratios of 50:50, 70:30, and 30:70 (w/w), similar trends were observed (Figure 1B). The cell viability decreased with increasing concentration, yet the formulation with the highest proportion of *C. gynandra* (70:30) showed the greatest viability across tested concentrations. Specifically, at concentrations ranging from 0.25–2 mg/mL, cell viability remained above 90%, which is considered non-cytotoxic based on prior standards. The formulations at 50:50 and 30:70 ratios also maintained viability above 90% at these concentrations but showed slightly decreased viability at higher doses. Literature supports these findings; Anbazhagi et al.[7] reported that higher concentrations of *C. gynandra* contain more flavonoids, which can induce cytotoxicity, while Kabir et al.[36] observed that *M. ptelefolia*, with high alkaloid content, can decrease cell viability and cause cell hypotonicity, shrinkage, and death. This information was instrumental in selecting the concentration range of 0.25–2 mg/mL for

subsequent anti-inflammatory assays, as these concentrations did not cause cytotoxicity in RAW 264.7 cells.

Anti-Inflammatory Activity of C. gynandra, M. ptelefolia, and Herbal Formulations

The anti-inflammatory potential was assessed via nitric oxide (NO) inhibition in lipopolysaccharide (LPS)-activated RAW 264.7 cells (Figure 2A and 2B). LPS activation induces macrophages to produce pro-inflammatory mediators, including NO, tumor necrosis factor-alpha (TNF-á), interleukins, and enzymes like cyclooxygenase-2 (COX-2). Diclofenac sodium, a nonsteroidal anti-inflammatory drug (NSAID), served as the positive control.

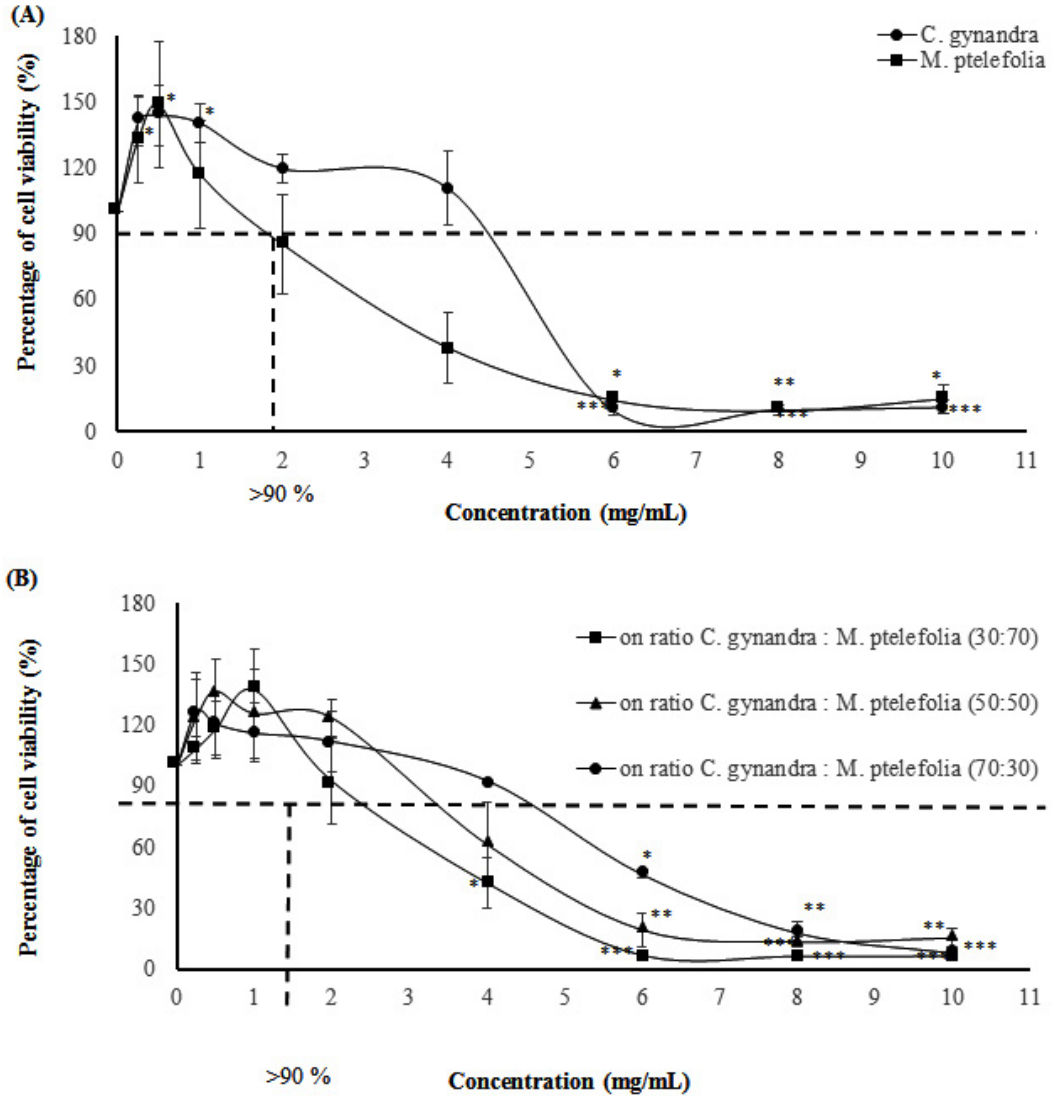

Fig. 1. Percentages of cell viability of RAW 264.7 cells after treated with the *C. Gynandra*, *M. Ptelefolia* and its herbal formulation as determined by MTT assay: (A) between single herb, *C. gynandra* and *M. ptelefolia* and (B) herb formulation, *C. gynandra*: *M. ptelefolia* at different ratios of 30:70, 50:50 and 70:30.
Data are expressed as mean ± SEM of three replicates (three independent experiments). Results were analysed by one-way ANOVA and followed by Dunnet tests. The means marked with *, **, *** are significantly different with *p*-value less than of 0.05, 0.01, and 0.001, respectively, as compared to untreated cells

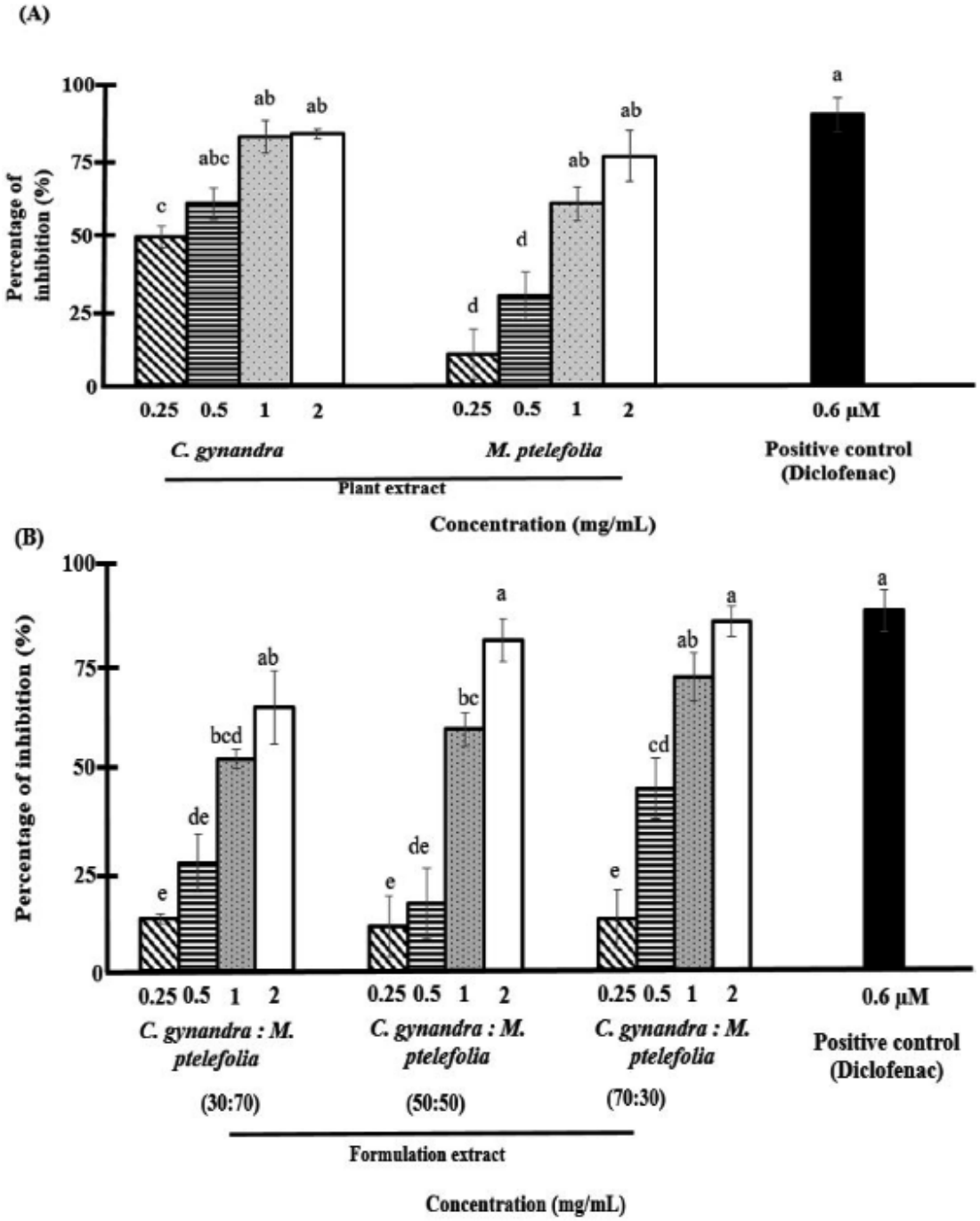

Fig. 2. Comparison of *C. gynandra*, *M. ptelefolia* and its herbal formulation (0.25–2 mg/mL) on the percentage of NO inhibition in LPS-induced RAW 264.7 cells: (A) between single herb of *C. gynandra* and *M. ptelefolia* and (B) herb formulation, *C. gynandra*: *M. ptelefolia* at different ratios of 30:70, 50:50 and 70:30. Data are expressed as mean ± SEM of three replicates (three independent experiment). Value with different superscript letters differ significantly. Data were analysed with one-way ANOVA followed by Tukey post-hoc test, ($P< 0.05$)

Table 1. Tentative identification of selected peaks from *M. ptefolia* and *C. gynandra* extracts

Peak number	T_R (min)	$[M-H]^-$ *m/z*	MS/MS *m/z*	Tentatively identified metabolites	Reference
			M. ptelefolia extract		
1	11.01	355.21	337, 209, 191, 163, 147, 129, 119	Coumaroylquinic hydrate	Harbaum et al., 2007 [49]
2	13.02	355.22	337, 209, 191, 172, 163, 144, 128, 119	Isomer of coumaroylquinic hydrate	Harbaum et al., 2007 [49]
3	16.58	325.22	163,145,133,117,69,59	*P*-coumaroyl glucoside	Nuengchamnong & Ingkaninan, 2009[50]
4	23.95	739.34	593, 575, 473, 429, 393, 327, 284, 255, 227, 199, 179, 151	Kaempferol neohesperidoside with rhamnose moiety	Sanchez-Rabaneda et al., 2003 [51]
5	24.37	739.38	593, 575, 547, 473, 393, 327, 284, 255, 227, 199, 179, 151	Isomer of kaempferol neohesperidoside with rhamnose moiety	Sanchez-Rabaneda et al., 2003 [51]
6	25.55	725.31	593, 575, 327, 284, 255, 277, 151	Kaempferol neohesperidoside with arabinose moiety	Sanchez-Rabaneda et al., 2003 [51]
7	25.83	755.32	723, 623, 605, 489, 314, 299, 285, 271, 243	Isorhamnetin-3-*O* rutinoside with arabinose moiety	Sanchez-Rabaneda et al., 2003 [51]
8	26.40	593.30	547, 447, 429, 363, 327, 284, 255, 227, 211, 151, 107	Kaempferol neohesperidoside	Sanchez-Rabaneda et al., 2003 [51]
9	26.90	609.40	315, 357, 300, 285, 271, 25, 243, 227, 151	Isorhamnetin glucoside with arabinose moeity	Sanchez-Rabaneda et al., 2003 [51]
10	28.17	593.32	547, 447, 429, 363, 327, 284, 255, 227, 211, 151, 107	Isomer of kaempferol neohesperidoside	Sanchez-Rabaneda et al., 2003 [51]
11	28.80	623.33	445, 357, 315, 299, 285, 271, 255, 243, 227, 150	Isorhamnetin-3-*O* rutinoside	Sanchez-Rabaneda et al., 2003 [51]
12	29.90	477.31	314, 229, 285, 271, 257, 243, 227, 214, 199	Isorhamnetin glucoside	Harbaum et al., 2007 [49]
13	30.90	579.27	447, 314, 299, 285, 271, 257, 243, 227, 187, 151	Isorhamnetin glucoside with 2 arabinose/xylose moieties	Sanchez-Rabaneda et al., 2003 [51]
			C. gynandra extract		
14	2.30	191.02	173, 111, 87, 85, 67, 57	Citric acid	Lacine et al., 2013 [52]
15	5.55	371.06	209, 191, 179, 173, 85	Caffeoylglucaric acid	Chen et al., 2014 [53]
16	7.40	339.07	225, 203, 177, 133	Esculetin glucoside	Simigiortis et al., 2015 [54]
17	8.13	297.06	179, 161, 135, 117, 89	Derivative of caffeic acid	Fang et al., 2002 [55]
18	9.63	369.05	207, 189, 127, 99, 83	Dimetoxycinnamic glucoside	Li et at., 2016 [56]
19	9.92	179.03	135, 117, 107, 89	Caffeic acid	Chen et al., 2014 [53]
20	11.83	353.04	207, 189, 127, 119, 99, 83	Dimethoxycinnamoyl coumaric acid	Li et al., 2016 [56]
21	12.84	383.06	207, 189, 127, 99, 83	Dimethoxycinnamoyl glucoronide	Li et al., 2016 [56]
22	13.80	609.15	343, 301, 179, 151	Quercetin rutinoside	Lacine et al., 2013 [52]
23	14.74	593.15	327, 285, 151	Kaempferol rutinoside	Lacine et al., 2013 [52]

Initial screening revealed that all extracts, including single herbs and herbal formulations, exhibited insignificant NO inhibition compared to diclofenac sodium. *C. gynandra* showed a dose-dependent increase in NO inhibition, reaching around 50% at 0.5 mg/mL, but the inhibition was not statistically significant beyond that point. Similarly, *M. ptelefolia's* NO inhibition was

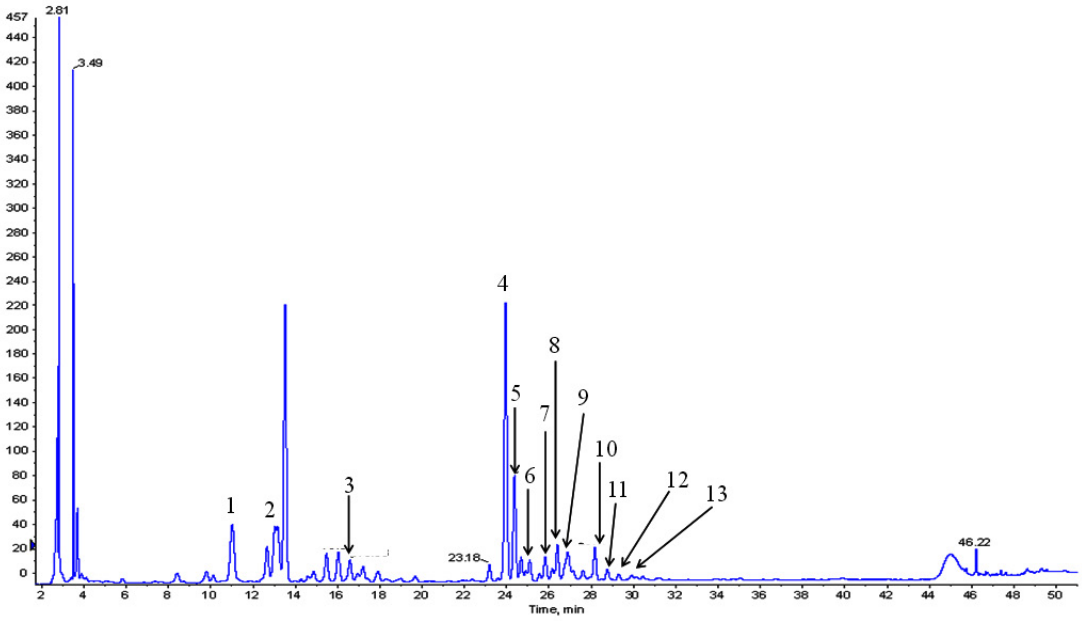

Fig. 3. HPLC chromatogram of *M. ptelefolia* extract at 280 nm absorbance. The numbering of selected peaks is in accordance to the tentative identification shown in Table 1

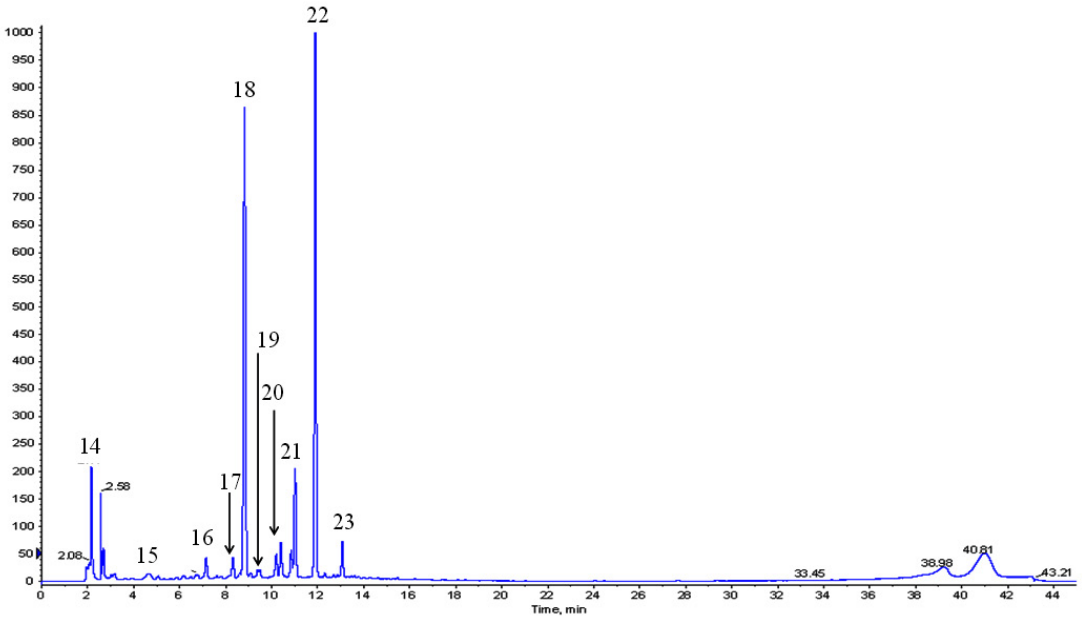

Fig. 4. HPLC chromatogram of *C. gynandra* extract at 280 nm absorbance. The numbering of selected peaks ais in accordance to the tentative identification shown in Table

insignificant starting from 1 mg/mL. The herbal formulations (30:70, 50:50, 70:30 ratios) exhibited increased NO inhibition with rising concentrations; however, at 0.5 mg/mL, their inhibitory effects were minimal and not significantly different from untreated controls. At higher doses (1 and 2 mg/mL), the formulations' NO inhibition approached that of diclofenac sodium, with the 70:30 formulation achieving approximately 65% inhibition at 2 mg/mL.

Comparison showed that single herb *C. gynandra* had superior NO inhibitory activity at 0.5 mg/mL, while the herbal formulations only matched the positive control at the highest tested concentrations (1 and 2 mg/mL). Overall, the data suggest that the herbal formulations do not significantly enhance NO suppression compared to *C. gynandra* alone, indicating a lack of synergistic effect under the tested conditions.

Identification of Metabolites and Evaluation of Synergism

Identification of metabolites from selected peaks was done to ascertain the possible metabolites that might contribute to the anti-inflammatory activities. A total of 13 and 10 metabolites (Numbers in parenthesis are peaks detected) were identified from *M. ptelofolia* and *C. gynandra* respectively (Table 1) . Figures 3 and 4 show the chromatograms of *M. ptelofolia* and *C. gynandra* corresponding to Table 1. Analysis from the identified metabolites indicates that both herbs are rich in flavonoids and its derivatives. In *M. ptelofolia* extract, metabolites (4, 5, 6, 8 and 10) were tentatively identified as kaempferol neohesperidoside and its derivatives, conjugated with either a rhamnose or arabinose sugars. Other flavonoids such as isorhamnetin glucoside conjugated with arabinose sugar (9), isorhamnetin-3-*O* rutinoside (11), isorhamnetin glucoside (12) and isorhamnetin conjugated with 2 arabinose or xylose sugars (13) were identified too. Coumaroylquinic hydrate (1), isomer of coumaroylquinic hydrate (2) and *P*-coumaroyl glucoside (3) were eluted out approximately at 11.01, 13.02 and 16.58 min respectively.

Quercetin rutinoside (22), an important flavonoid in *C. gynandra* extract was identified at 13.8 min. The presence of quercetin rutinoside or rutin as one of the main marker compound in *C. gynandra* was validated in a documented patent by Msika et al.[48] Another flavonoid, kaempferol rutinoside (23) was detected after the elution of rutin. Phenolics from the cluster of hydroxycinnamic acids were found abundantly in *C. gynandra* extract too. Caffeic acid (19) and its derivative (17), dimethoxycinnamic glucoside (18), dimethoxycinnamoyl coumaric acid (20) and dimethoxycinnamoyl glucoronide (21) were some of the hydroxycinnamic acids identified. Other phenolics such as citric acid (14), caffeoylglucaric acid (15) and esculetin glucoside (16) were tentatively identified as well.

The naturally occurring phenolic acids in plants have always been associated with their health benefiting properties. Presence of kaempferol and its derivative in *M. ptelefolia* extract could explain the high inhibition activity of nitric oxide production. Kaempferol was shown to reduced significantly inducible nitric oxide synthase (iNOS) protein level in a concentration-dependent manner and as well the mRNA of iNOS.[57] The same pattern was observed in our study where the RAW 264.7 cells were induced using LPS, as to mimic the induction of NOS. When the concentration of *M. ptefolia* extract was increased from 0.25 to 2.0 mg/mL, the production of NO ceased significantly. On the other hand, high concentration of quercetin rutinoside could lead to possible anti-inflammatory activity in *C. gynandra* extract. Lee and colleague[58] demonstrated on how quercetin rutinoside attenuated various pro-inflammatory mediators in LPS induced RAW 264.7 cells. Besides, hydroxycinnamic acids such as dimethoxycinnamic derivatives and caffeic acid in *C. gynandra* have potent anti-inflammation property too, as indicated by Gunasekaran and colleague.[59]

Even though both herbs possessed significant anti-inflammatory activity, mixing their extracts in certain ratio does not necessarily enhance their activity as shown in this study. The dogma of 'two better than one' in herbal extract formulations follows the logic of interaction between the phytochemicals in formulated extracts.[60] When the formulated herbal extracts have better activity compared to the individual ones, the net effect is said to be synergistic. The contrary to the synergism is known as antagonistic when the formulated herbal extracts have poor activity than the individual extracts. However,

when the activity of herbal extract formulation is not significant compared to the individual ones, additive term will be used instead.[61] An additive effect was observed in all formulations at 2 mg/mL compared to their respective individual extracts in inhibiting NO (Figure 2). However, the interaction pattern changes to antagonistic when the concentrations were reduced from 1 mg/mL to 0.25 mg/mL. None of the formulations had better efficacy in inhibiting NO at 0.25 mg/mL compared to single extract of *C. gynandra* which registered at 50% inhibition.

DISCUSSION

The present study provides a comprehensive evaluation of the cytotoxic and anti-inflammatory properties of *C. gynandra, M. ptelefolia*, and their herbal formulations, with results supported by phytochemical profiling. The cytotoxicity assays confirmed that at concentrations ranging from 0.25 to 2 mg/mL, the extracts and formulations are safe for RAW 264.7 macrophages, aligning with prior research indicating the safety of herbal extracts at moderate doses. Notably, *C. gynandra* demonstrated higher cell viability across tested concentrations compared to *M. ptelefolia*, which suggests that *C. gynandra* contains fewer cytotoxic constituents or that its bioactive compounds are less damaging to macrophage cells. These findings are consistent with earlier reports where flavonoids such as quercetin and phenolic acids like caffeic acid, prevalent in *C. gynandra*, are known to exert anti-inflammatory effects without significant cytotoxicity at moderate doses.

The observed decrease in cell viability at higher concentrations (>4 mg/mL for *C. gynandra* and >1 mg/mL for *M. ptelefolia*) can be attributed to the high content of bioactive phytochemicals, particularly flavonoids and alkaloids, which at elevated doses can induce oxidative stress, cell membrane disruption, or apoptosis. Anbazhagi et al.[7] indicated that flavonoids, while generally beneficial, can exert cytotoxic effects at high concentrations, possibly due to pro-oxidant activity. Similarly, Kabir et al.[36] reported that alkaloids in *M. ptelefolia* could cause cell hypotonicity and shrinkage, leading to cell death, especially at higher doses. These findings reinforce the importance of dose optimization in herbal medicine research to maximize therapeutic efficacy while minimizing toxicity[37].

The anti-inflammatory activity assessed via NO inhibition revealed that C. gynandra possesses a more potent effect compared to *M. ptelefolia*, especially at lower concentrations (0.5 mg/mL). Although none of the extracts or formulations showed statistically significant inhibition compared to diclofenac sodium, the trend suggests that *C. gynandra*'s phytochemicals are more effective in modulating macrophage inflammatory responses[38]. This aligns with phytochemical profiling that identified key bioactive compounds such as quercetin rutinoside (rutin) and hydroxycinnamic acids, both well-documented for their anti-inflammatory mechanisms—namely, downregulation of iNOS, suppression of pro-inflammatory cytokines, and inhibition of nuclear factor-kappa B (NF-êB) activation.[39]

The LC-MS/MS analysis provided critical insights into the phytochemical composition underlying these activities. In *M. ptelefolia*, flavonoids like kaempferol neohesperidoside and its derivatives, conjugated with sugars such as rhamnose and arabinose, are known to inhibit nitric oxide synthase activity. Kaempferol, in particular, has been shown to significantly reduce iNOS expression, thereby decreasing NO production, a key mediator in inflammatory processes. The detection of isorhamnetin derivatives further supports anti-inflammatory potential, as these compounds are also recognized for their ability to modulate inflammatory signaling pathways.[40] These phytochmicals maybe nanoencapsulated to improve their efficacy. [41-43]

In *C. gynandra*, the presence of quercetin rutinoside (rutin) and kaempferol rutinoside, along with phenolic acids like caffeic acid and its derivatives, underscores its anti-inflammatory capacity. Quercetin rutinoside has demonstrated efficacy in attenuating pro-inflammatory mediators by inhibiting enzymes such as COX-2 and inducible nitric oxide synthase, and suppressing cytokines like TNF-á and IL-6[43,44]. Hydroxycinnamic acids like caffeic acid are potent antioxidants and have been shown to inhibit NF-êB activation, thus reducing inflammation. These phytochemicals may act synergistically to produce anti-inflammatory effects, although the exact interactions require further elucidation.

Despite the promising individual activities, the combination of *C. gynandra* and *M. ptelefolia* in herbal formulations did not produce synergistic effects in NO inhibition. Instead, the interaction pattern was predominantly additive at the higher concentration tested (2 mg/mL), implying that the combined effects simply sum up without amplification. At lower concentrations (0.25–1 mg/mL), the interactions shifted toward antagonism, where the formulations exhibited less NO inhibition than the individual extracts. This phenomenon can be explained by phytochemical interactions, where certain compounds may compete for the same targets or interfere with each other's absorption, distribution, or cellular activity. For example, flavonoids and alkaloids could potentially inhibit each other's binding to inflammatory mediators or modulate enzyme activity in opposing ways.

The lack of observed synergism underscores the complexity of herbal interactions and the importance of ratio optimization. While traditional medicine often relies on synergistic effects, modern scientific validation indicates that not all combinations produce enhanced efficacy. The phytochemical profile suggests that specific compounds like quercetin rutinoside and kaempferol derivatives are primarily responsible for anti-inflammatory effects; however, their interactions within complex mixtures can vary depending on concentration, extraction method, and the presence of other constituents.

Furthermore, this study highlights the necessity for detailed mechanistic studies to understand how phytochemicals interact at molecular levels. For instance, future investigations could examine the effects of these extracts on key inflammatory signaling pathways such as NF-êB, MAPK, or STAT, and explore their effects on cytokine profiles beyond NO, including TNF-á, IL-1â, and IL-6[45]. Additionally, exploring different ratios, extraction techniques, or formulations (e.g., nanoemulsions, encapsulation) might reveal conditions under which synergistic effects become apparent[46-47].

CONCLUSION

This study assessed the cytotoxicity and anti-inflammatory potential of *Cleome gynandra* and *Melicope ptelefolia* leaf extracts, both individually and in combination using RAW 264.7 macrophage cells. Among the two, *C. gynandra* exhibited higher cell viability and lower cytotoxicity, indicating its greater safety profile. In nitric oxide (NO) inhibition assays, *C. gynandra* showed moderate activity, while *M. ptelefolia* and their combinations demonstrated limited inhibitory effects, particularly at lower concentrations.

LC-ESI-MS/MS metabolite profiling revealed several bioactive compounds in both extracts. *C. gynandra* was rich in quercetin, kaempferol, and gallic acid, all known for their potent anti-inflammatory and antioxidant activities through modulation of nitric oxide synthase and suppression of pro-inflammatory cytokines. *M. ptelefolia* primarily contained scopoletin, auraptene, and rutin, which are associated with inhibition of COX-2 and inflammatory mediators. The presence of these compounds supports the traditional use of both plants in treating inflammatory conditions.

However, the combination of *C. gynandra* and *M. ptelefolia* at the tested ratios (especially 70:30 w/w) did not show a synergistic improvement in anti-inflammatory activity, highlighting the complexity of herb-herb interactions. These findings underscore the need to better understand phytochemical compatibility and optimize formulation ratios in herbal product development.

In conclusion, *C. gynandra* demonstrates greater potential as a standalone anti-inflammatory agent compared to *M. ptelefolia*, primarily due to its richer content of flavonoids and phenolic acids. Future research should focus on refining extract ratios, exploring detailed molecular mechanisms of action, and validating these effects through in vivo studies to support the development of safe and effective plant-based anti-inflammatory therapeutics.

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Conflict of Interest

The authors do not have any conflict of interest.

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This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

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Not Applicable.

Author Contribution's

Muhammad Aiman Haiqal bin Ismail – original draft, Formal analysis, Data curation; Koh Soo Peng– Supervision, Formal analysis, Funding acquisition, Conceptualization; Sanimah Simoh – LC-ESI-MS/MS Profiling; Armania Nurdin – Supervision, Formal analysis; Machap Chandradevan – LC-ESI-MS/MS Profiling; Muhammad Nazrul Hakim – Supervision, Formal analysis, Project administration.

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