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Short Communication

Inhibition of inducible nitric oxide production by *Caryota urens* and its active constituents umbelliferone and rutin

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ABSTRACT

Inducible Nitric Oxide Synthase (iNOS) is a key mediator in a variety of diseases, and thus it is interesting to discover new iNOS inhibitors. This study aimed to evaluate if the hydroalcoholic leaf extract of *Caryota urens* (CULHA) consisting of rutin and umbelliferone prevents inflammation in response to external stimuli. To demonstrate the therapeutic potential of CULHA against inflammatory diseases, nitric oxide production (NO) mediated by iNOS was evaluated. In lipopolysaccharide induced RAW 264.7 cells, CULHA, rutin and umbelliferone exhibited remarkable inhibition of NO at 61%, 30% and 41% respectively without affecting the cell viability at the highest concentration tested. In conclusion, results obtained in this work substantiate the traditional uses of this plant and brings a brief understanding of their anti inflammatory potential.

1. Introduction

Nitric oxide synthase (NOS) is an isoenzyme that catalyses the synthesis of NO from L-Arginine in the presence of NADPH. Multiple cofactors inclusive of calmodulin (CaM), tetrahydrobiopterin (BH4), Heme, and flavins (FMN and FAD) and various other cofactors are involved in the process [1]. There are three well characterized isoforms of NOS: Neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS), [2]. iNOS is a type II NOS which is Ca^{2+} dependent and its expression in inflammatory cells increases in response to a specific stimuli. In the presence of stimuli such as lipopolysaccharide (LPS), macrophages are capable of producing sustained and high levels of Nitric oxide (NO) mediated by iNOS from arginine [3].

The overexpression of iNOS is one of the direct consequences of an inflammatory process. An increased level of iNOS expression is observed in a number of inflammatory disorders such as rheumatoid arthritis, crohn's disease and inflammatory bowel diseases leading to detrimental effects [4]. Because of its role and importance in mediating inflammatory diseases, iNOS enzyme has emerged as a key target in finding new drugs/inhibitors as anti-inflammatory agents.

Historically, medicinal plants remain as an important source of lead molecules that play a critical role in drug discovery. However, research work by various groups suggests that medicinal plants are capable of accumulating heavy metals which can lead to toxicity [5,6]. To demonstrate that the plant is devoid of such toxic compounds, MTT assay was performed.

Meanwhile, effective screening techniques are employed in parallel for the rapid screening of potential hit compounds. Based on these ideas, the current study was performed to investigate whether CULHA, umbelliferone and rutin can inhibit iNOS by employing Griess reagent assay. Previous study by our group showed the presence of rutin and umbelliferone in leaf hydroalcoholic extract of *C.urens* (CULHA) [7]. HPLC quantification of rutin and umbelliferone is shown in Table 1 (supplementary).

2. Materials and methods

2.1. Preparation of Caryota urens leaf hydroalcoholic extract

C.urens used in this study was collected from VISTAS campus, Chennai in December, 2016. It was authenticated (PARC/2016/3315) by plant taxonomist Dr. J. Jeyaraman, PARC, Tambaram, Chennai-600045.

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Collected leaves were washed with water to remove dirt and dried under shade for 10 days. Dried leaves were cut into small pieces and 200 g of it was soaked in 70% ethanol for 72 h with continuous stirring using orbital shaker [8,9]. The extract was filtered using whatmann filter paper No.1 and further concentrated by distillation at 60 °C. The plant extract obtained was then stored at 4 °C until when needed.

2.2. Sample preparation

Rutin and Umbelliferone were purchased from Sigma Aldrich. 50 mg/ml stock of Rutin, Umbelliferone and *C.urens* leaf exract (CULHA) was prepared in DMSO. Samples prepared were filtered through 0.22 micron filter.

2.3. Preparation of LPS

LPS was purchased from Sigma (L3129-10 MG). This was extracted from *Escherichia coli* serotype O127:B8 and strain ATCC 12740. 5 mg/mL stock of LPS was prepared with water, filtered through 0.22 micron filter and stored at −20°c until required.

2.4. Cell culture

RAW 264.7 is a murine macrophage cell line purchased from NCCS Pune, India. Cells were grown in DMEM medium supplemented with FBS and incubated at 37 °C in 5% CO_2 in an air humidified chamber.

2.5. MTT assay

Determination of Cell viability was performed under aseptic conditions according to the method of Scuderio et al., 1988 [9]. Briefly, RAW 264.7 macrophages at 5×10^4 cells/well were seeded into 96 well cell culture plate. Rutin, umbelliferone or CULHA was added to the respective wells at a concentration of 12.5, 25, 50, 100 and 200 μg/mL. 20 μL of DMEM was added to the control well. After 24 h of incubation, the wells were administered with 20 μL of 5 mg/ml MTT dye solution followed by incubation for 4 h. Cell culture media was carefully removed by aspiration without disturbing the cells and 200 μL of DMSO was added to each well to dissolve formazan. The absorbance of the purple colour developed was read at 570 nm. The percentage of viable cells was calculated as below:

Percentage Viability = (OD sample−OD blank) / (OD control−OD blank) × 100 [10].

2.6. Measurement of nitrite concentration in the culture media

Inflammation is a highly coordinated process that is mediated by macrophages/monocytes [11]. In response to immune challenge such as LPS, macrophages induce the production of NO. In this present investigation, LPS stimulated RAW 264.7 cells were used as target for assessing the anti-inflammatory potential of CULHA and its active constituents rutin and umbelliferone. The protocol was performed under aseptic condition at the laminar air flow cabinet. Cells (1.5×10^4 cells/well) were cultured in DMEM medium without phenol red for 24 h. Rutin, umbelliferone or CULHA was added to the respective well at the concentrations of 3.12, 6.25 and 12.5 μg/mL. 20 μL of DMEM was added to the control well. After 2 h of incubation, 1 μg/mL of LPS was then added to the corresponding wells and cells were re-incubated for further 18 h. The nitrite concentration were measured in the culture supernatant as an index of NO production according to Griess reaction [12].

To 100 μl of supernatant collected, 100 μl of Griess reagent (Sigma, G4410) was added and incubated in dark for 10 min. Sodium nitrite was used as a standard. The intensity of the red pink color developed was read at 540 nm. Nitrite concentration was determined and compared with standard sodium nitrite.

Percentage NO inhibition = (NO level of control cells - NO level of treated cells)/NO level of control cells × 100.

3. Results and discussion

3.1. The cytotoxicity of CULHA and its active constituents on RAW 264.7 cells

CULHA and its active constituents umbelliferone and rutin were screened to assess toxicity by performing the MTT assay in order to confirm the cytotoxic effect in RAW 264.7 cells and also to determine the effective concentrations for further *in vitro* anti-inflammatory assays. Results of the cytotoxicity are shown in Fig. 1A. CULHA exhibited no significant cytotoxicity against the cell line. On the contrary, pure compounds umbelliferone and rutin exhibited moderate cytotoxic activity against the RAW 264.7 cell line.

Percentage cell viability (CV) was calculated. 90% cell viability was observed at a concentration of 12.5 μg/mL for umbelliferone and rutin. CULHA expressed similar results at 25 μg/ml at which the concentration of rutin and umbelliferone were 17.5 ng/ml and 4.8 ng/ml respectively, calculated by means of gram equivalence from HPLC data. Previous study by different groups [13,14] suggests that synthetic flavonoids and coumarins exhibit higher cytotoxic activity and more interestingly they are known to inhibit tumor cell growth via apoptosis mechanism. Consistent to these findings, umbelliferone and rutin screened in our study showed decrease in cell viability with moderate cytotoxic activity, the effect being dose dependent. On the contrary, treatment of RAW 264.7 cells with CULHA did not induce pronounced cytotoxic effect. Decreased cytotoxic activity of the extract could be attributed to the lower concentrations of rutin and umbelliferone in it or the presence of other phytochemicals.

Observation of morphological changes in the cell line treated with extract/individual compounds rutin and umbelliferone was done to monitor the changes caused. These involved changes in cell size such as cell shrinkage and formation of apoptotic bodies were noted. At concentrations above 100 μg/mL the cells became small, rounder and detached from the surface of the wells denoting cell death.

Further assays were planned at concentrations below 12.5 μg/ml for CULHA and its active constituents owing to their 90% cell viability.

3.2. CULHA and its active constituents inhibited NO production in macrophages

It has been established that LPS stimulation is capable of inducing NO production in murine macrophage RAW 264.7 cells and is therefore used as a target in the investigation of molecular mechanisms of a potential anti-inflammatory drug [15]. Hence, LPS induced macrophage is used as a rapid system for screening of drugs and the subsequent screening of potential inhibitors against NO production. Many medicinal plants have time and again proven their value as inhibitors of NO production in LPS-activated macrophages. Thus, plants or plant derived compounds possessing strong inhibitory activity against NO production may have promising therapeutic potential in the treatment of inflammation [16].

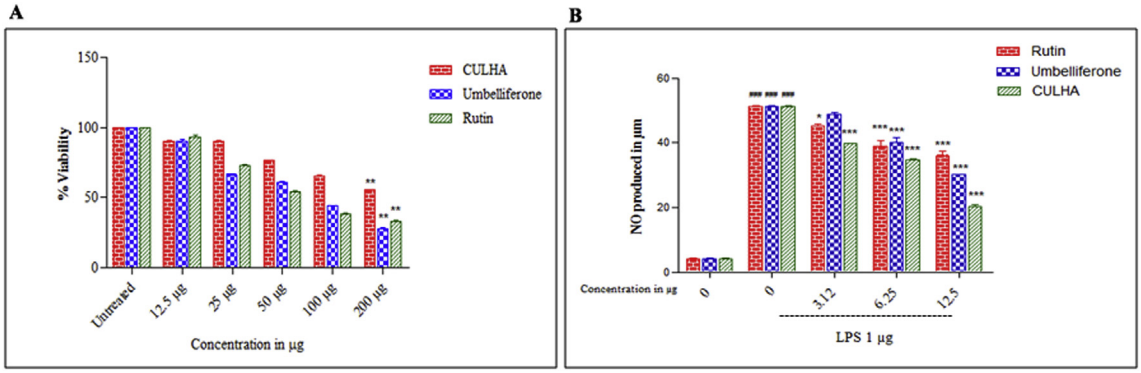

Fig. 1. A. Cytotoxic evaluation of CULHA and its active constituents umbelliferone and rutin. Mean value of triplicates is represented. Mean differences in all the groups was calculated using one-way analysis of variance (ANOVA). **$p < 0.05$ vs control. B. Effect of CULHA and its active constituents umbelliferone and rutin (3.12 µg, 6.25 µg and 12.5 µg) on production of NO. Mean value of triplicates is represented. Mean differences in all the groups was calculated using one-way analysis of variance (ANOVA). ***$p < 0.05$ vs. LPS-treated control group; $^{###}p < 0.05$ vs. control.

The incubation of RAW 264.7 cells with CULHA, rutin and umbelliferone at various concentrations (3.125, 6.25 and 12.5 µg/mL) significantly suppressed the production of NO in a dose dependent manner in comparison to the LPS control group. As shown in Fig. 1B, the basal nitrite levels were (1.48 µM) in the control group. Upon stimulation with 100 ng/mL of LPS, the production of NO rose significantly to levels of 51.2 µM. At 12.5 µg/mL, CULHA showed the greatest NO scavenging effect of 61% followed by rutin and umbelliferone at 30% and 41% respectively. Similar results by previous study [17] shows that rutin reduced nitric oxide levels by 46% at 24 µg/ml.

At 12.5 µg/mL, CULHA had 2.4 ng of umbelliferone and 8.8 ng of rutin, calculated by means of gram equivalence from HPLC data. Even at the presence of lowest concentration of umbelliferone and rutin, CULHA at 12.5 µg/ml had significant anti inflammatory potential against the pure compounds used at same concentration in the study. This suggests a synergistic effect of several phytocompounds present in the extract. These results recommended that the extract CULHA, which inhibits NO production may have potential role in the management of inflammatory diseases.

4. Conclusion

Our experiments showed that umbelliferone, rutin and CULHA inhibited LPS-induced activation of NO in macrophages. The results of MTT cell viability assay revealed that the inhibitory effect of the extract and compounds was not due to cell damage (viability > 90%). More specifically, our data demonstrates that *C.urens* is more potent than umbelliferone and rutin, suggesting a synergistic interaction between the major compounds and other possible minor compounds. To our knowledge, the results demonstrated for the first time the anti-inflammatory potential of *C.urens*, thus validating its traditional use. Based on these results, CULHA can be developed as a new therapeutic agent against inflammatory diseases, although high activity of *C.urens in vitro* needs to be confirmed by further *in vivo* investigations.

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Conflict of interest

None declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaim.2020.09.002.

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