ELUCIDATION OF ANTI-INFLAMMATORY ACTIVITY OF A NEW CYCLIC ALKALOID COMPOUND FROM ROOT BARK OF ZIZIPHUS NUMMULARIA (AUBREV.): IN VITRO, IN SILICO AND IN VIVO STUDIES

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ABSTRACT
Inflammatory diseases present a significant burden to global health, often requiring targeted and specific therapeutic agents for effective management. Ziziphus nummularia (Aubrev.), a traditional medicinal plant, offers a potential source for these agents. This article aims to explore the anti-inflammatory potential of a novel cyclic alkaloid compound isolated from the root bark of Ziziphus nummularia. The cyclic alkaloid compound, ([16-methoxy-10-(3-methyl-butyryl)-2-oxa-6, 9, 12-triaza-tricyclo [13.3.1.03, 7] nonadeca-1(18), 13, 15(19), 16-tetraene-8, 11-Dione]), exhibited promising anti-inflammatory properties. The potential of this isolated compound (IC) was evaluated in vitro by measuring the levels of nitric oxide (NO), prostaglandin-E2 (PGE2), and tumor necrosis factor-alpha (TNF-α). Additionally, Absorption, Distribution, Metabolism, and Excretion (ADME) simulations and molecular docking to the TNF-α receptor were carried out for IC. In vivo evaluations of both the IC and its ethanolic extract (EE) were performed using carrageenan-induced paw oedema and arachidonic acid/xylene-induced ear oedema tests. The IC showed a higher inhibition of TNF-α compared to other compounds, reaching a maximum inhibition of 88.00% at a concentration of 50.11 μM. In silico analysis revealed that IC formed hydrogen bonds with residues, aspartate at position 45 (ASP 45) and glutamine at position 47 (GLN 47). The IC significantly attenuated oedema induced by carrageenan, xylene, and arachidonic acid. Hence, this compound may offer a potential therapeutic approach for treating inflammation in future clinical applications.

Keywords: Anti-inflammatory agents; Molecular docking; Nitric oxide; Oedema; Prostaglandins; Tumor necrosis factor-alpha, Ziziphus nummularia

INTRODUCTION
Inflammation is an intricate physiological process orchestrated by a plethora of molecular mediators that regulate and modulate the immune response of body to tissue injury or pathogens (Cekici et al., 2000; Qian, 2017; Siegfried et al., 2020). This immunopathological reaction can be attributed to the overabundance of specific mediators present in macrophages (Davies and Taylor, 2015; Locati et al., 2020). Key among these mediators is tumor necrosis factor-alpha (TNF-α), interleukins (IL), nitric oxide (NO), and prostaglandins (PG) (Montgomery and Bowers, 2012; Vignali and Kuchroo, 2012; Zelova and Hosek, 2013). Each of these molecules plays a pivotal role in instigating and perpetuating the inflammatory cascade (Habib and Ali, 2011; Ricciotti and Fitzgerald, 2011; Allerton et al., 2018). However, when their regulatory mechanisms are compromised, leading to overproduction or prolonged activity, they can foster a range of pathological conditions. From a therapeutic standpoint, targeting and diminishing the activity or concentration of these mediators offers a potential avenue for curbing the exacerbation and progression of inflammation. The Rhamnaceae family encompasses the species Ziziphus nummularia (Aubrev.), commonly known as wild jujube. Indigenous to India’s semi-arid and dry regions, this plant has been traditionally employed as an herbal remedy by the native populations for a myriad of health complaints including inflammation, cough, cold, diarrhea, dysentery, and pain (BACHAYA et al., 2009; UPADHYAY et al., 2011; BISWAS et al., 2017). Notably, the anti-inflammatory potential of its leaves, as well as those of related species, has been documented in prior research (SOLIMAN, 2011; GOYAL et al., 2012,2013). While various molecules have been derived from the plant’s leaves and fruits, the root bark remains relatively uncharted territory in the scientific literature. However, it is noteworthy to mention that our group had previously identified a triterpene derivative from the root bark, exhibiting both anticancer and anti-inflammatory activities (Dey Ray and Dewanjee, 2015; Dey Ray et al., 2015). In the arena of drug discovery, the technological advent of in silico techniques has revolutionized our understanding of drug-receptor interactions, underpinning rational drug design. Particularly, understanding the mechanistic pathways through which novel drugs manifest anti-inflammatory efficacy is paramount. Within this purview, molecular docking has emerged as a computational technique of significant interest, facilitating insights into potential drug-receptor affinities. Given this context, the present study embarked on a comprehensive exploration to isolate, elucidate the structure, and discern the anti-inflammatory mechanism of a novel cyclic alkaloid compound (referred to as IC) from the root bark of Z. nummularia (Aubrev.). This study was structured in four phases. The maiden phase revolved around the isolation of the new chemical entity and subsequent structural determination. Subsequent in vitro analyses ascertained the bioactivity of this isolated compound. In the following phase, an in silico approach was employed, probing the interaction dynamics of IC with a key inflammatory mediator, TNF-α. The concluding phase encompassed in vivo assessments, evaluating the therapeutic implications of IC in a murine model. Concurrently, the therapeutic potential of the ethanolic extract (EE) was determined and juxtaposed against the bioactivity of IC. Employing an integrative approach combining in vitro and in silico methods, this study thus aimed to shed light on the complete understanding of the anti-inflammatory mechanism of IC, focusing on its interaction dynamics with TNF-α.

This molecular insight complements traditional knowledge and prior discoveries related to the therapeutic potential of Z. nummularia. The in vivo evaluations, in conjunction with the in vitro and in silico experiments, present a comprehensive understanding of the anti-inflammatory efficacy of the IC.
The effect of IC on the acute toxicity of EE and IC was estimated by oral administration of IC for 2 weeks. The study on the acute toxicity of EE and IC were performed according to Lorke (1983). The detailed procedure is given in Supplementary Figures.

Carrageenan-induced oedema of paw in mice
Carrageenan-induced paw oedema in mice is a useful approach for evaluating the anti-inflammatory potential of active phytoconstituents (Dordovic et al., 2007). Mice were starved for 12 hours prior to the experiment. Sixty mice were divided into six groups (n=10). Group 1 received tween-80 (1 %) orally, 30 minutes ahead of carrageenan injection. This group acted as inflammation control. Groups 2 and 3 orally received 100 mg/kg and 200 mg/kg EE, respectively. Group 4 as well as Group 5 orally received 400 μg/kg and 600 μg/kg of IC, respectively. Group 6 was given aspirin (10 mg/kg) and acted as positive control. Following carrageenan administration, paw oedema was measured at 0 hour, 1 hour, 3 hours, 4 hours, and 5 hours.

Xylenol/araachidonic acid-produced oedema of ear
For each model, mice were divided into six groups (n=10). Inflammation was generated in mice by applying 30 μl of following reagents: arachidonic acid 0.1 mg/ml in acetone (Young et al., 1984) and xylenol (Nunez Guillen et al., 1997) on inner and outer faces of right ear. Group 1 received tween-80 (1 %) orally, 30 minutes ahead carrageenan injection. This group acted as inflammation control. Groups 2 and 3 orally received 100 mg/kg and 200 mg/kg EE, respectively. Groups 4 and 5 orally received 400 μg/kg and 600 μg/kg of IC, respectively. Group 6 was given aspirin (10 mg/kg) and acted as positive control. Following carrageenan administration, paw oedema was measured at 0 hour, 1 hour, 2 hours, 3 hours, 4 hours, and 5 hours.

Statistical analysis
Results were presented as mean ± standard error of mean (SEM). The impact of the values was checked by student t test using computerized GraphPad InStat (version 3.05), GraphPad software, USA. Differences were considered statistically significant at p<0.05.
RESULTS

Preliminary structural characteristics of the isolated compound (IC)

The fractions numbered 49 to 56, obtained from column chromatography, displayed a predominant spot with minor impurities when analyzed on thin-layer chromatography (TLC) using silica gel plates. The visualization of these spots was achieved by treating the plates with Dragendorff’s reagent. To remove the observed impurities, these fractions were washed with hexane. Following the washing, fractions 49 to 56 were combined and consolidated. A subsequent purification step involved another hexane wash. From this process, a total yield of 0.075 grams of the compound was obtained.

The purified, bioactive eluent was further subjected to high-performance thin-layer chromatography (HPTLC) for detailed analysis. Detection was carried out at a wavelength of 450 nm. The mobile phase used for the chromatography consisted of a solvent system: methanol, ethyl acetate, chloroform, and formic acid in the ratio of 1:7:2:0.2. The retention factor (Rf) value of the compound was found to be 0.59. Additional HPTLC data included a peak height of 2.8 and an area under the peak measuring 111 (Supplementary Figure 1).

The melting point (m.p) of the purified compound was determined to be in the range of 465-468 °C. An elemental analysis was performed on the isolated compound with the molecular formula C8H13N2O. The calculated percentage composition was Carbon (C), 65.17%; Hydrogen (H), 7.55%; Nitrogen (N), 10.86%; and Oxygen (O), 16.54%.

This approach and the subsequent data helped in the isolation and preliminary structural elucidation of the compound of interest.

The isolated compound showed following spectral characteristics:

1HNR (CDCl3): δ 4.336 (d, J=6.0 Hz, H-3), 171.196 (-CH=, C-1 / 16), 77.017 (CH, C-3), 39.176 (CH2, C-4 / 5), 55.874 (-CH, C-10), 125.432 (-CH2, C-10’-1’, 10’-2’), 29.706 (CH, C-10’-3’), 14.771(-CH, C-10’-4’), 118.17 (CH=, C-13 / 14), 128.766 (CH-15), 60.369 (-OCH3, O-CH3), 113.674 (CH, C-17 / 18 / 19) (Supplementary Figures 2-5).

IR (KBr) νmax, 2955, 2869, 1668, 1468, 3065, 3394, 1221, 1033, 883, 1454, 1376 cm⁻¹ (Supplementary Figure 7).

MS (FAB): m/z: 387. (M+1) (Supplementary Figure 8).

Table 1 Pharmacokinetic prediction of the isolated compound (IC) by QikProp® 3.2.

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<tr>
<th>Sl. No.</th>
<th>Descriptor</th>
<th>Description</th>
<th>Recommended Range</th>
<th>Predicted Value of IC</th>
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<td>1</td>
<td>mol_MW</td>
<td>Molecular weight of the molecule</td>
<td>130.0-725.0</td>
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<tr>
<td>2</td>
<td>SASA</td>
<td>Total solvent accessible surface area (SASA) in square angstroms using a probe with a 1.4 Å radius</td>
<td>300.0-1000.0</td>
<td>645.87</td>
</tr>
<tr>
<td>3</td>
<td>FOSA</td>
<td>Hydrophobic component of the SASA (saturated carbon and attached hydrogen)</td>
<td>0.0-750.0</td>
<td>456.87</td>
</tr>
<tr>
<td>4</td>
<td>FISA</td>
<td>Hydrophilic component of the SASA (SASA on N, O and hydrogen on heteroatom)</td>
<td>7.0-330.0</td>
<td>94.90</td>
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<tr>
<td>5</td>
<td>PISA</td>
<td>Τ (carbon and attached hydrogen) component of SASA</td>
<td>0.0-450.0</td>
<td>93.66</td>
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<tr>
<td>6</td>
<td>volume</td>
<td>Total solvent-accessible volume in cubic angstroms using a probe with a 1.4 Å radius</td>
<td>500.0-2000.0</td>
<td>1203.93</td>
</tr>
<tr>
<td>7</td>
<td>donorHB</td>
<td>Estimated number of hydrogen bonds that would be donated by the solute to water molecules in an aqueous solution. Values are averages taken over a number of configurations, so they can be non-integer</td>
<td>0.0-6.0</td>
<td>2.25</td>
</tr>
<tr>
<td>8</td>
<td>acceptHB</td>
<td>Estimated number of hydrogen bonds that would be accepted by the solute to water molecules in an aqueous solution. Values are averages taken over a number of configurations, so they can be non-integer</td>
<td>2.0-20.0</td>
<td>7.25</td>
</tr>
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<td>9</td>
<td>QPlogP o/w</td>
<td>Predicted octanol / water partition coefficient</td>
<td>2.0-6.5</td>
<td>3.92</td>
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<tr>
<td>10</td>
<td>Human oral absorption</td>
<td>Predictive qualitative human oral absorption. The assessment uses a knowledge-based set of rules, including checking for suitable values percent human oral absorption, number of metabolites, number of rotatable bonds logP; solubility and cell permeability</td>
<td>1.2, 3 for low, medium and high absorption respectively</td>
<td>3.0</td>
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<tr>
<td>11</td>
<td>% human oral absorption</td>
<td>It predicts human oral absorption on 0 to 100% scale. The prediction is based on a quantitative multiple linear regression model. This property usually correlates well with human oral absorption.</td>
<td>&gt;80% is high ≤25% is poor</td>
<td>80.65</td>
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<td>12</td>
<td>#rtFG</td>
<td>This particular descriptor indicates the number of reactive functional groups. The presence of these groups can lead to decomposition, reactivity, or toxicity problems in vivo.</td>
<td>0 to 2.0</td>
<td>0.0</td>
</tr>
<tr>
<td>13</td>
<td>CNS</td>
<td>Predictive central nervous activity on a –2 (inactive) to +2 (active) scale.</td>
<td>-2.0 to 2.0</td>
<td>0.0</td>
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<tr>
<td>14</td>
<td>Lipinski’s rule of five</td>
<td>Lipinski’s rules of five are: mol_MW &lt; 500, QPlogP o/w &lt; 5, donorHB ≤ 5, acceptHB ≤ 10. Compounds that satisfy these rules are considered drug like. (The “five” refers to the limits, which are multiples of 5).</td>
<td>Maximum is 4</td>
<td>Satisfy the Lipinski’s rule of five</td>
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</table>

In vitro study

Dose-dependent inhibition of NO and TNF-α production by IC in LPS/IFN-γ stimulated RAW 264.7 cells

Treatment of cells (RAW 264.7) with LPS/IFN-γ was found to increase the concentrations of NO, TNF-α, and PGE2 (Figure 2). The effect of IC on NO production was dose-dependent, with the highest inhibition being 67.00 percent at 5018.72 nM (~50.11 μM). PGE2 inhibition, on the other hand, did not appear to be dose-dependent, with the maximum inhibition of 55.00 percent noted at 100 nM. IC also had a concentration-dependent effect on TNF-α production. The highest inhibition was 88.00 percent at 50.11 μM. IC had the most inhibitory potential on TNF-α production when compared to NO. As a result, TNF-α receptor was used in molecular docking.
Molecular docking of ligand IC with TNF-α receptor: interaction with residues ASP 45 and GLN 47

In the molecular docking study, the ligand IC established two distinct hydrogen bonds with the amino acid residues of the protein target, TNF-α receptor. Specifically, the residues ASP 45 and GLN 47 were involved in this interaction (Figure 3). The oxygen atom within the linkage of IC functioned as a hydrogen bond acceptor, forming bonds with the side chains of both GLN 47 and ASP 45. In contrast, the –NH group present in the pyrrolidine ring of IC acted as a hydrogen bond donor.

In silico study

Physicochemical and ADME computational analysis of IC in relation to its drug-likeness and interaction with TNF-α receptor

The physicochemical properties of the IC were evaluated to ascertain its drug-likeness. Molecular weight (MW), QPlogP (which is a measure of the partition coefficient between octanol and water), the number of hydrogen bond donors (donorHB), and the number of hydrogen bond acceptors (acceptHB) were examined with respect to Lipinski's rule of five. The estimated values for these parameters fell within the designated acceptable ranges, indicating that IC exhibits properties consistent with known drug molecules (Lipinski et al., 2001). Further computational assessments were conducted to gain insights into the physicochemical characteristics of IC. The solvent accessible surface area (SASA), which are denoted as FOSA and FISA respectively, were calculated to be 456.87 and 94.90. Additionally, the π component of the SASA, termed as PISA, was found to be 93.66. The overall volume of the IC was computed to be 1203.93. Such measurements underscore the importance of physicochemical characteristics of IC in relation to its drug-likeness and interaction with TNF-α receptor.

Figures and Tables

Figure 2 Effect of the isolated compound (IC) on nitric oxide (NO), prostaglandin-E2 (PGE2), and tumor necrosis factor-alpha (TNF-α) production to demonstrate its inflammatory inhibition potential.

Figure 3 Three-dimensional (3-D) view of docking pose of minimum energy structure complex of the isolated compound (IC) (PDB ID: 1A8M) viewed using Glide XP visualizer of Schrödinger Maestro. Hydrogen bonds are shown as yellow dash and bonded with ASP 45 and GLN 47.

Figure 4 Docked pose of the isolated compound (IC) complemented by the hydrophilic-hydrophobic interface of the TNF-α receptor.

Figure 5 Effect of isolated compound (IC) and ethanolic extract (EE) on carrageenan produced oedema of paw in mice. *p < 0.05 compared with control group.

In vivo study

Acute toxicity study

In male mice, the LD₅₀ doses of EE and IC were determined to be 3020 mg/kg body weight (bw) and 2200 mg/kg bw, respectively.
Effect of EE and IC on carrageenan-produced oedema of paw in mice

A well-known and well-established paradigm for acute inflammation is carrageenan-induced inflammation. During the 4th and 5th hours after carrageenan therapy, oral doses of EE at 100 mg/kg and 200 mg/kg were found to reduce inflammation. The percentage inhibitory activities were 20.0 and 24.4, respectively (p < 0.05), as compared to control. At the 3rd, 4th, and 5th hours after receiving a 400 μg/kg oral dose of IC, the percentage inhibitions were 31.82, 35.56, and 33.33, respectively (p < 0.05). At the same incubation time, percentage inhibitions at 600 μg/kg of IC were 36.36, 40.00, and 37.78, respectively (p < 0.05). The percentage inhibitions were 38.64, 42.22, and 40.00 (p < 0.05) after the 3rd, 4th, and 5th hours respectively, following aspirin therapy at an oral dosage of 10 mg/kg. The data show that at 4 hours after carrageenan therapy, the percentage inhibition of IC at the aforementioned two doses and aspirin at a dose of 10mg/kg began to decrease. In the case of EE, however, the percentage inhibition remained constant between the 4th and 5th hours (Figure 5).

Effect of EE and IC on xylene/arachidonic acid-produced oedema of ear

Inhibition of xylene-induced ear oedema inflammation by EE at 100 mg/kg and 200 mg/kg was 19.51 and 31.71 percent (p < 0.05), respectively. For the arachidonic acid-induced model, the inhibitions were 27.06 and 30.59 percent, respectively (p < 0.05). The suppression of xylene-induced ear oedema inflammation by IC at 400 and 600 μg/kg was 50.00 and 53.66 percent, respectively (p < 0.05). The inhibitions of both models were 57.32 and 61.18 percent, respectively (p < 0.05) after aspirin at a dosage of 10 mg/kg (Figure 6).

DISCUSSION

The primary objective of this study was to isolate and characterize an active compound from the bark of Z. nummularia (Aubrev.), to determine its molecular structure, and to explore its mechanism of action using in vitro, in silico, and in vivo methods. The isolated compound, denoted as IC, is [(16-methoxy-10-(3-methylbutyl)-2-oxa-6, 9, 12-triaza-tricyclo [13.3.1.03, 7] nonadeca-1(18), 13, 15(19), 16-tetraene-8, 11-Dione). To the best of our knowledge, this study marks the first documentation of its bioactivity.

NO and PGE2 are prominent mediators synthesized by macrophages (Lin et al., 2014; Shi et al., 2014). Previous literature suggests that TNF-α acts as an endogenous mediator in the inflammatory process (Parameswaran and Patial, 2010; Ye et al., 2016). The inflammation process is exacerbated by the overproduction of NO, PGE2, and TNF-α, leading to the damage of neighbouring cells and tissues. Consequently, molecules that can attenuate the levels of these inflammatory mediators are prospective candidates for anti-inflammatory agents.

Molecular docking studies provided a simulated environment to evaluate the interaction between IC and the TNF-α receptor. Amino acid residues involved in this interaction included LYS 90, ASP 45, GLN 27, ARG 131, GLU 23, GLY 24, GLN 47, GLU 135, GLU 25, LEU 26, and ASN 46 (Figure 7).

Figure 6 Effect of the isolated compound (IC) and ethanolic extract (EE) on xylene/arachidonic acid-produced oedema of ear. *p < 0.05 compared with control group.

Having established the anti-inflammatory potential of IC in vitro and supported this finding with in silico data elucidating its interaction with TNF-α, the present study proceeded to validate its efficacy in vivo. The study employed a carrageenan-induced inflammation model and another model using a combination of xylene and arachidonic acid. The carrageenan test, known for its sensitivity, induces a two-phase oedema in mice due to the surge of inflammatory mediators like NO. The inflammation process is exacerbated by the overproduction of NO, tumour necrosis factor-alpha (TNF-α), and prostaglandin E2 (PGE2), suggesting the observed anti-inflammatory action of IC might stem from the suppression of PGE2 and/or NO synthesis. In the presence of xylene, the release of pro-inflammatory mediators from sensory neurons, mast cells, and other immune cells is facilitated (Figure 8).

Figure 7 The key amino acid shown within the active site of TNF-α comprising LYS 90, ASP 45, GLN 27, ARG 131, GLU 23, GLY 24, GLN 47, GLU 135, GLN 25, LEU 26, and ASN 46.

Figure 8 Efficacy of IC as an anti-inflammatory compound, both in cell cultures and animal models, possibly by interacting with the TNF-α receptor and inhibiting the production or effects of specific inflammatory mediators. (A) The anti-inflammatory efficacy of IC shown in vitro on RAW 264.7 cells when treated with lipopolysaccharide/interferon-gamma (LPS/IFN-γ). LPS/IFN-γ treatment leads to an increase in nitric oxide (NO), tumour necrosis factor-alpha (TNF-α), and prostaglandin E2 (PGE2) in cells. IC demonstrates an ability to inhibit NO and PGE2 production. IC has been observed to inhibit TNF-α production dose-dependently and most profoundly. As a consequence of these observations, molecular docking experiments were initiated with the TNF-α receptor; (B) In silico data concerning the interaction between IC and the TNF-α receptor indicates that IC has potential binding affinity with the TNF-α receptor thereby inhibiting its functions; (C) Validating anti-inflammatory potential of IC in vivo, two distinct prostaglandins and inherently slow reactions. Additionally, arachidonic acid regulates NO production and serves as a precursor to PGE2, suggesting the observed anti-inflammatory action of IC might stem from the suppression of PGE2 and/or NO synthesis. In the presence of xylene, the release of pro-inflammatory mediators from sensory neurons, mast cells, and other immune cells is facilitated (Figure 8).
inflammation mice models were employed: the first incorporated carrageenan-induced paw oedema, and the latter integrated both xylene- and arachidonic acid-induced ear oedema. Thus, IC is a promising anti-inflammatory compound, showcasing efficacy both in cell-based assays and animal models. This potential can possibly be attributed to the interaction of IC with the TNF-α receptor and its ability to curtail the production or effects of specific inflammatory mediators.

CONCLUSIONS

The compound IC, a novel cyclic alkaloid extracted from the bark of Z. nummularia (Aubrev.), demonstrated significant anti-inflammatory properties. Our findings reveal that IC effectively mitigated the expression of inflammatory mediators like NO and TNF-α and exhibited a pronounced reduction in carrageenan, xylene, and arachidonic acid-induced oedema. This compound shows promising potential as a therapeutic agent for inflammation management in future clinical applications. The comprehensive, multi-pronged approach adopted in this research evaluates the in vitro anti-inflammatory effects of the compound by measuring key inflammatory markers. Additionally, in silico pharmacokinetic analyses and interaction studies are conducted, complemented by thorough in vivo evaluations. This comprehensive approach emphasizes a rigorous scientific evaluation of the therapeutic potential of the compound, bridging traditional knowledge with contemporary scientific methodologies.

Declaration of interest: The authors declare no conflict of interest.

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REFERENCES


Supplementary Figure 1: High-performance thin-layer chromatography (HPTLC) profiling of the isolated compound (IC).

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Supplementary Figure 2: $^1$H nuclear magnetic resonance ($^1$H NMR) spectroscopy of the isolated compound (IC).

Supplementary Figure 3: $^1$H nuclear magnetic resonance ($^1$H NMR) spectroscopy of the isolated compound (IC).
Supplementary Figure 4: $^1$H nuclear magnetic resonance ($^1$H NMR) spectroscopy of the isolated compound (IC).

Supplementary Figure 5: $^1$H nuclear magnetic resonance ($^1$H NMR) spectroscopy of the isolated compound (IC).

Supplementary Figure 6: $^{13}$C nuclear magnetic resonance ($^{13}$C NMR) of the isolated compound (IC).

Supplementary Figure 7: Fourier-transform infrared spectroscopy (FTIR) spectroscopy of the isolated compound (IC).

Supplementary Figure 8: Mass spectroscopy of the isolated compound (IC).
Supplementary Figure 9 High resolution mass spectroscopy of the isolated compound (IC).