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Research paper

2-Methyl-pyran-4-one-3-*O*-β-D-glucopyranoside isolated from leaves of *Punica granatum* inhibits the TNFα-induced cell adhesion molecules expression by blocking nuclear transcription factor-κB (NF-κB)

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ABSTRACT

Here, we report bioactivity-guided isolation, purification and characterization of a novel compound, 2-methyl-pyran-4-one-3-*O*-β-D-glucopyranoside (MPG) from the leaves of *Punica granatum*. The structure of MPG was established on the basis of its detailed spectral analyses. We demonstrated that MPG not only inhibited the expression of cell adhesion molecules but also significantly blocked its functional consequence, that is, the adhesion of neutrophils on human endothelial cells monolayer. To elucidate the molecular mechanism of action of MPG, we showed that MPG decreased the transcript levels of ICAM-1, VCAM-1 and E-selectin genes. Using electrophoretic mobility shift assay (EMSA) and western blot analyses, we demonstrated that MPG significantly blocked both the TNFα-induced translocation and activation of nuclear transcription factor-κB (NF-κB). Thus, MPG could be useful as a novel lead molecule for developing future anti-inflammatory agents.

1. Introduction

Inflammation, an integral part of immune surveillance mechanism, is a complex response to local injury and involves various immune cells and numerous inflammatory mediators. Accumulation of leukocytes at the site of an inflammatory response or bacterial invasion is crucial for immune surveillance mechanism [1]. Endothelial cells lining the blood vessels are important players in the recruitment of leukocytes at the site(s) of inflammation. Inflammatory cytokines like IL-lβ, TNFα and bacterial lipopolysaccharide (LPS) induce the activation of Cell Adhesion Molecules (CAMs), such as ICAM-1 (intercellular cell adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1) and E-selectin on endothelial cells. The increased expression of CAMs "activate" the endothelium to become adhesive for peripheral blood leukocytes [2–6].

Once the leukocytes have migrated across the endothelial barrier into the site of injury, additional chemoattractants stimulate them to release peroxides and proteases which function to destroy the offending pathogen [7]. Thus, leukocyte recruitment is critical for host defense [8,9]. In addition to being involved in host defense, excessive leukocyte–endothelial interactions lead to barrier dysfunction and increased permeability in a variety of inflammatory conditions like chronic obstructive pulmonary disease (COPD), asthma, acute respiratory distress syndrome (ARDS), glomerulonephritis, ulcerative colitis, psoriasis and autoimmune vasculitis [10,11]. The molecules that could block these interactions have been targeted as potential therapeutic treatments for acute and chronic inflammatory diseases [12].

Several anti-adhesion therapies, like use of specific monoclonal antibodies (mAbs), have been found to be beneficial for controlling various diseases [13]. However, due to endotoxin contamination, unpredictable clinical manifestations, such as secondary antibody formation, cellular activation, and other complications like serum sickness and anaphylaxis, the practical use of mAbs is limited [14]. Keeping in view the redundancy of the immunological network, blocking a single cell adhesion molecule may not offer much benefit.

Numerous medicinal herbs have been shown to modulate specific cellular and humoral immune responses [15]. The pomegranate

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(*P. granatum*) is a fruit-bearing deciduous shrub or small tree is widely cultivated throughout Asia and tropical Africa. Extracts of all parts of the fruit appear to have therapeutic properties [16] and some studies report the bark, roots and leaves of the tree have anti-oxidant, anti-inflammatory, anti-carcinogenic medicinal benefits as well [17–20]. Unique tannins occur in pomegranate leaves, as well in peel. Leaves of *P. granatum* also contain glycosides of apigenin, a flavone with progestinic [21] and anxiolytic [22] properties. The fresh leaves extract of *P. granatum* has been found to be associated with free-radical scavenging activity [23]. Ellagic acid, another major ellagitannin present in the leaves showed anti-oxidant and anti-inflammatory properties [24,25]. Apigenin, a flavone, and Punicalgin, an ellagitanin, also present in the leaves of *P. granatum* have been known to exhibit anti-inflammatory actions [16].

Although *P. granatum* and its constituents have been implicated in many different biological activities, there is no study investigating the potential of its leaves extracts on the inhibition of expression of cell adhesion molecules on human endothelial cells. Here we describe for the first time bioactivity-guided isolation, purification and characterization of a novel compound 2-methyl-pyran-4-one-3-*O*-β-D-glucopyranoside (MPG) from the *n*-butanol extract of *P. granatum* leaves and report its inhibitory effect on TNFα-induced expression of cell adhesion molecules on human umbilical vein endothelial cells (HUVECs). We also showed that MPG blocked the activation and translocation of nuclear transcription factor-κB (NF-κB).

2. Results and discussion

2.1. Inhibition of ICAM-1 expression-guided isolation and purification of an active principle from the leaves of P. granatum

A schematic chart has been presented to illustrate the steps followed to isolate and purify the active principle from leaves of *P. granatum* (Fig. 1). The crude ethanolic extract prepared from freshly collected leaves of *P. granatum* was tested for its cytotoxicity on human umbilical vein endothelial cells (HUVECs) (Fig. 2). The maximum tolerable concentration of the crude extract was found to be 150 μg/ml (Fig. 2A). The crude extract was then assessed for its ability to inhibit the TNFα-induced ICAM-1 expression on HUVECs. The results showed that it significantly inhibited the expression of ICAM-1 (55%), VCAM-1 (57%) and E-selectin (51%) on HUVECs at a non-toxic maximum tolerable concentration of 150 μg/ml (Fig. 2B). The crude extract was also screened at this concentration for its ability to inhibit the adhesion of neutrophils to the endothelium and was found to exhibit 56% inhibition (Fig. 2B).

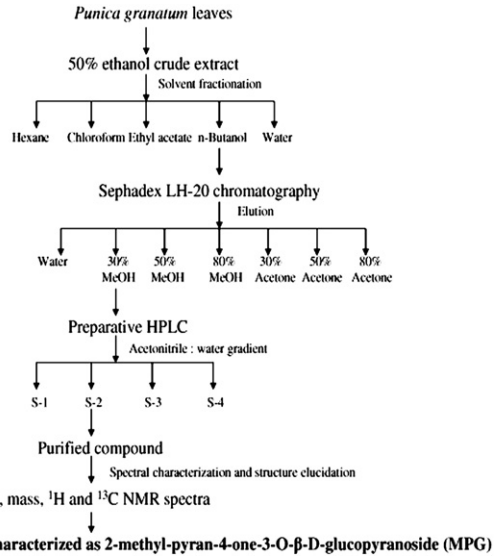

Fig. 1. Purification chart for the bioactivity-guided isolation and purification of a novel compound, MPG, from leaves of *Punica granatum*.

Thus, the crude extract was subjected to further purification through solvent fractionation into hexane, chloroform, ethyl acetate, n-butanol and water soluble fractions (Fig. 1). The cytotoxicity of each solvent fraction was tested to determine their respective maximum tolerable concentrations (Fig. 2C) followed by assessment of their ICAM-1 inhibitory activities. The fractions were further tested for their ability to inhibit the adhesion of neutrophils onto endothelial cells monolayer. The *n*-butanol fraction was found to be the most active in inhibiting the TNFα-induced expression of ICAM-1 (68% inhibition) and adhesion of neutrophils (70% inhibition) onto the endothelium at a maximum tolerable concentration of 100 μg/ml (Fig. 2D).

The active n-butanol fraction was further subjected to purification onto a Sephadex LH-20 column as described in Fig. 1. The cytotoxicity of each column fraction was tested and the maximum tolerable concentration of all the column fractions was found to be 100 μg/ml (Fig. 3A). This was followed by assessment of their inhibitory activities at a maximum tolerable concentration of 100 μg/ml as described in Fig. 3B. Amongst all the fractions, 30% methanol fraction was found to exhibit maximum inhibitory activity (Fig. 3B).

The active 30% methanol fraction was subjected to preparative high-performance liquid chromatography (HPLC) (Fig. 3C). The HPLC analysis showed four peaks which were collected and tested for their cytotoxicity (Fig. 3D). The maximum tolerable concentration of all the HPLC peak fractions was found to be 100 μg/ml (Fig. 3D). This was followed by assessment of their ICAM-1 and neutrophil adhesion inhibitory activities (Fig. 3E). The HPLC Peak S-2 was found to be the most active fraction with maximum inhibitory activity (Fig. 3E).

2.2. Characterization of purified active principle from P. granatum leaves

The structure of the purified active principle was determined by performing ESI MS, IR, ^{1}H NMR and ^{13}C NMR including 2D NMR spectroscopy. The IR spectrum of the identified compound showed strong absorptions at 1647 cm^{-1} indicating the presence of a C=O group. ESI MS showed molecular ion peak at m/z 311.37 $[M + Na]^{+}$ which is consistent with the formula $C_{12}H_{16}O_8$. The ^{1}H NMR of the compound showed two doublets (each for ^{1}H) centered at δ 8.14 ($J = 5.6$ Hz) and δ 6.43 ($J = 5.6$ Hz) thus indicating the presence of a 2,3 substituted pyran ring (Fig. 4A). Apart from these signals, there was a three proton singlet at δ 2.36 for methyl group in the ^{1}H NMR spectrum, which confirms the substitution at pyran ring. There was a doublet for one proton at δ 4.17 ($J = 7.2$ Hz) which could be assigned to the α-anomeric proton of D-glucopyranose (H-1′). This is corroborated by the ^{13}C NMR spectra which exhibited the characteristic hexose signals at δ 61.70, 70.17, 77.50, 77.81 and 102.61. Thus, the data from ^{1}H and ^{13}C NMR along with the aid of 2D NMR techniques such as Heteronuclear Multiple Bond Coherence (HMBC) (Fig. 4A), Heteronuclear Multiple Quantum Coherence (HSQC) and Nuclear Overhauser Effect Spectroscopy (NOESY) interactions (Supplementary material) suggested that the purified active principle from *P. granatum* leaves could be characterized as 2-methyl-pyran-4-one-3-*O*-β-D-glucopyranoside (MPG) (Fig. 4B). The compound has been compared with the authentic specimen synthesized in our laboratory and the spectra (IR, NMR) are found to be identical in all respect. This novel compound, MPG, was found to be isolated for the first time from *P. granatum* leaves.