# Anti-inflammatory effects of *zingiber officinale roscoe* involve suppression of nitric oxide and prostaglandin E2 production

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## Abstract

**Background and objective:** Inflammation is a physiological response to injury and infection. However, chronic inflammation causes tissue damage and is a feature of most chronic diseases. Despite significant progress in developing therapies to target chronic inflammation over the years, almost all current therapies have serious side effects. The current investigation is to identify naturally-existing anti-inflammatory therapies with fewer side effects.

**Methods:** The anti-inflammatory effects and mechanisms of action of extracts and fractions obtained using vacuum liquid chromatography (VLC) from ginger (*Zingiber officinale*) on the production of nitric oxide (NO) and prostaglandin E2 ($PGE_2$) were investigated. NO and $PGE_2$ production were induced by stimulating the mouse RAW264.7 monocyte/ macrophage cell line with lipopolysaccharide (LPS). Levels of NO and $PGE_2$ were determined using the Griess method and enzyme linked sorbent assay (ELISA), respectively.

**Results**: Extracts of two Zingiber officinale species obtained with chloroform showed potent inhibitory effects on NO and PGE2 production. The extracts had a higher potency than N(G)-nitro-L-arginine methyl ester (L-NAME), a known specific inducible nitric oxide synthase (iNOS) inhibitor and were comparable in their effects on PGE2 with Indomethacin, a specific PGE2 inhibitor. Further, we identified a fraction (F6) that had most potent inhibitory effects.

**Conclusion:** The study shows that extract of *Zingiber officinale* have strong inhibitory effects on key pro-inflammatory mediators involved in chronic inflammation. Both the extracts and F6 had better inhibitory effects than established pharmaceutical inhibitors of NO and $PGE_2$.

**Keywords:** Zingiber officinale roscoe**,** inflammation, nitric oxide, prostaglandin $E_2$

## Introduction

Inflammation is a complex physiological response to infections, injury and damaged tissues that mediates the healing process [1].Chronic inflammation itself drives pathological processes leading to diseases such as allergy, atherosclerosis, autoimmune diseases and cancer. Inflammation is, therefore, finely tuned to protect from infections and injury while preventing its excesses from causing chronic diseases. For over half a century, increased understanding of mechanisms and mediators of inflammation have directly contributed to the discovery of large and successful clinical application of numerous anti-inflammatory drugs. However, almost all current pharmaceutical drugs have serious side effects [2]. The current investigation was carried out to explore the effect and mechanism of action of naturally-existing compounds in zingiber officinale roscoe with therapeutic effects on inflammation. In this study we examine the effect and

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potential mechanism of action of two varieties of ginger, a plant widely used as an anti-inflammatory medication in traditional medicines in many parts of the world [3]. NO is a short-lived free radical with a range of physiological and pathophysiology effects [4]. Besides NO synthesis, arachidonic acid metabolism is also upregulated during inflammation. In response to a variety of nonspecific stimuli, phospholipase $A_2$ induces the production of arachidonic acid from membrane phospholipids which are further catalysed by cyclooxygenase (COX-1/2) leading to the production of prostanoids such as $PGE_2$, $PGD_2$ and thromboxane (Tx) $A_2$. During bacterial infections, monocytes and macrophage are stimulated to produce $PGE_2$ and interleukin 12 (IL12). Bacterial LPS and IL1β stimulate COX-2 transcription via NF-κB transcription factor activation leading to inflammatory cascades that promote T cell activation and differentiation [5]. Subsequently, monocytes/ macrophage-derived PGE2 acts as paracrine signals to activate dendritic cells (DC) resulting in activation which migrate to lymph nodes to serve as antigen-presenting cells (APCs) upon encounter with T cells. As T lymphocyte activation is a key factor in chronic inflammatory diseases, we opted to study the effects of ginger, ginger extracts and fractions on the generation of NO and $PGE_2$ production by activated macrophages. Ginger is a versatile plant which has been used as a spice and a herbal medicine for over 3000 years 6, 7. Ginger belongs to the genus Zingiber family of the Zingiberaceae that is under the order Zingiberales and tribe Zingibereae. Worldwide, there are estimated to be about 1,200 species of Zingiberacea which belong to 18 genera. Many medicinal ginger preparations are used as traditional medicine, for example as post-partum medication, post-natal care treatment, arthritis and psoriasis 8. Although there are some reports on the anti-inflammatory effects of the ginger species Z. officinale Roscoe sp. (ZORT) 9, 10, however, the exact mechanism of action is not properly studied. In the current investigation the optimal extraction methods for the anti-inflammatory components of common ginger and also the ZORT species are studied.

## Methods

### Preparation of plant extracts

Extracts from 2 types of ginger species, Z. officinale Roscoe var rubrum Theilade (ZORT) and Z. officinale Roscoe (common ginger) were obtained and studied for comparing their anti-inflammatory effects. The rhizome of the two ginger species studied were first collected, washed and dried in a drier for 48 hours at 40±5°C, then grounded into powder and stored at 4°C until used. The extraction efficiency of anti-inflammatory components of the plants of 3 solvents, chloroform, ethanol and water were compared. The extracts obtained by maceration, using these 3 solvents for ZORT were chloroform labeled as HB02, ethanol HB03 and water HB04. Extracts from common ginger were obtained with chloroform (HC02), ethanol (HC03) and water (HC04). The anti-inflammatory effects of the extracts on NO and PGE2 production by the RAW264.7 macrophage cell line stimulated with 0.1µg/ml of LPS (method is described in more detail in the next sections) were compared. The suppressive effects on NO production by extracts obtained using water, HB04 and HC04 were significantly inferior to those obtained with chloroform and ethanol and were, therefore excluded from further analysis. Both extracts were chemically-profiled by capillary electrophoresis (HP 3D -CE). This approach is based on separating ionic species according to their charge and frictional force in a small capillary filled with electrolyte driven by electroosmotic flow 11. The analysis was carried out in polyimide-coated fused silica capillaries

(TSP100375) with a length of 40cm and internal diameter of 50µm. In order to identify the nature of therapeutic constituents, the extracts were further fractionated using vacuum liquid chromatography (VLC). This method separates groups of active compounds according to a gradient of non polar : polar solvent ratios. In brief, 10-40g of ZORT extract was subjected to VLC on silica (Silica gel 60 PF254+366, Merck, Nottingham, UK) and 21 fractions were collected from gradients of a hexane: ethyl acetate mixture. The collected fractions were evaporated in a rotary evaporator and concentrated fractions left to dry at room temperature before testing.

**Assessment of anti-inflammatory properties of ginger extracts**

The potential anti-inflammatory effects of the extracts and 21 fractions obtained from the VLC fractionation were determined by assessing their effects on suppressing NO and PGE2 production by macrophages. The mouse macrophage cell line (RAW264.7) was used for these studies. Briefly, RAW264.7 cells were cultured in DMEM medium (BioWhittaker, Cologne, Germany) supplemented with 10% foetal bovine serum (FBS) (Gibco-Invitrogen, Paisly, UK) and 100U/ml penicillin and 100U/ml streptomycin at 37oC with 5% CO2 in the air until used for the experiments NO and PGE2 production by 2.5x105 RAW264.7 cells following stimulation with lipopolysaccharide (LPS) (Sigma-Aldrich, Gillingham, UK) at 0.1µg/ml were measured.

**Measurement of NO**

The NO content of supernatants from the cultured RAW264.7 cells was determined using the Griess method by measuring nitrite ($NO_2^-$), a stable breakdown product of NO. One hundred µl of culture supernatants were dispensed into 96 well plates followed by the addition of 100µl of Griess reagent (mixture of 1 % sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylenediamine, all from Sigma-Aldrich, in distilled water). NO content was then quantified by change in colour (absorbance at 560nm) within 10 minutes using a microplate reader (TECAN GENios). Nitrite content was calculated by extrapolation from a standard curve constructed using a range of known concentration of sodium nitrite up to 100µM.

**Measurement of PGE2**

The concentration of PGE2 in culture supernatants of stimulated RAW264.7 cells was determined using a PGE2 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Abingdon, UK). The concentration of PGE2 was extrapolated from a standard curve constructed for optical density values obtained at 450nm for known inputs of PGE2 standard from 2500-39pg/ml. One hundred and fifty µl of sample, control or PGE2 standard dilutions were added to each well of the ELISA plate which was coated with goat anti-mouse polyclonal antibody. Then, 50 µl of primary monoclonal antibody to PGE2 was added into each well except for the no sample blank (NSB) and the plate incubated for 1 hour at room temperature on horizontal orbital shaker at 500±50 rpm. Later on, 50 µl of PGE2 conjugated with alkaline phosphatase was added and plate was incubated for 2 hours on the shaker. The wells were then aspirated and washed four times with wash buffer, blotted on clean and 200 µl substrate added and plate further incubated for 30 minutes. Finally, 100 µl of stop solution was added and the OD was determined at 450nm using microplate reader (TECAN, GENios). PGE2 production was calculated by extrapolation using the curve constructed for the PGE2 standard using curve fit program (GRAPHPAD).

## Results

**Assessment protocols for the efficiency of prepared extracts from ginger plants.**

The anti-inflammatory effects of the extracts on NO and $PGE_2$ production by the RAW264.7 macrophage cell line stimulated with 0.1µg/ml of LPS were compared. The suppressive effects on NO production by extracts obtained with chloroform, both HB02 and HC02, were significantly more effective in inhibiting NO production by the stimulated macrophage line than the ethanol extracts Figure 1. All extracts whether with chloroform or ethanol were more effective at the same concentration than L-NAME ($p<0.001$), a specific inhibitor of NO production.

**Ginger extracts efficiently inhibited PGE2 production.**

HB02 had the most potent inhibitory effects on PGE2 production by the stimulated cell line Figure 2.. The inhibitory effect of HB02 was higher than those of HB03, HC02 and HC03 although the differences were not statistically significant due to variation in inhibition obtained from different experiments. The inhibitory effects of HB02, in contrast, were highly consistent. When the inhibitory effects of the extracts were compared with indomethacin at same concentration (10µg/ml) they were comparable particularly that of HB02, which almost similar to indomethacin.

**Fractionation of HB02 and identifying fractions with most potent anti-inflammatory effects.**

Twenty one fractions were collected and each faction was screened for their potency in inhibiting NO production by LPS-stimulated RAW 264.7 cells. The fractions were assessed at 20µg/ml (approximately the $IC_{50}$ obtained for HB02) and the % inhibition of NO production compared with stimulated RAW 264.7 cells determined. The data showed that fractions 5, 6, 7 and 10 (F5, F6, F7 and F10, respectively) inhibited NO production by LPS-stimulated RAW 264.7 cells by >80% Figure 3. The inhibitory effects of these fractions were higher than unfractionated HB02 and also L-NAME (used at 50µg/ml). In particular F5 and F6 exhibited the highest inhibitory effects on NO production by the stimulated RAW 264.7 cells. This observation was consistent as indicated by the relatively low SEM values obtained for the repeat experiments. In contrast, the least potent fractions were F13, F14 and F15. Based on these results, the fractions were then re-tested to identify their exact IC50 to confirm the data on NO and PGE2 inhibition. These data confirm the results shown in Figure 3 and confirmed that F6 had the lowest IC50 of all fractions Figure 4.

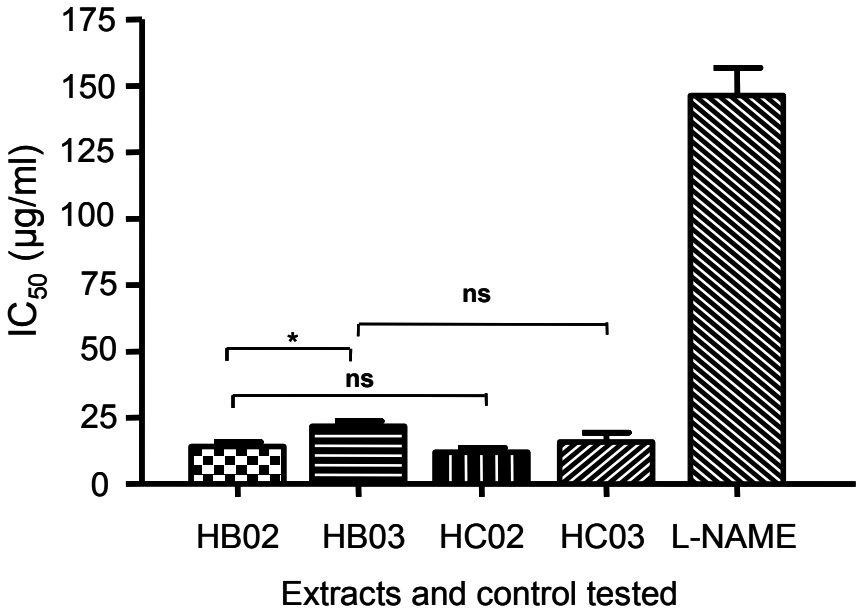


**Figure 1:** Median inhibitory concentration ($IC_{50}$) of different extract of ginger and L-NAME on NO production. Four extract preparations, two each from ZORT (HB02 and HB03) and common ginger (HC02 and HC03) were compared for their inhibitory effects on NO production by RAW 264.7 cells stimulated with 0.1µg/ml LPS

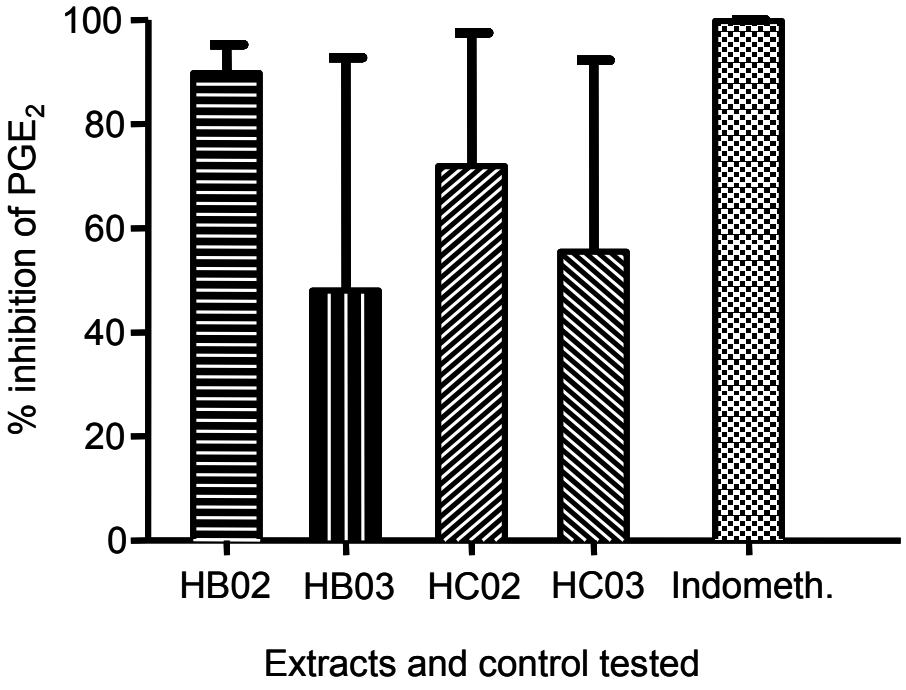


**Figure 2:** The inhibitory effects of four extract preparations (HB02, HB03, HC02 and HC) from ZORT and common ginger in addition to that of indomethacin on $PGE_2$ production. The extracts and indomethacin were used at fixed concentrations of 10µg/ml.

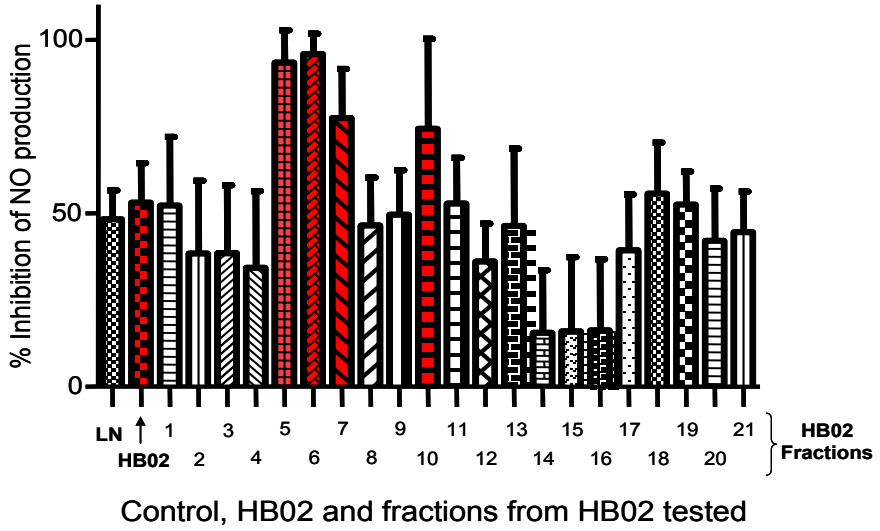


**Figure 3:** Effects of the HBO2 extract from ZORT and its 21 fractions on NO production. The fractions were tested at 20µg/ml for their ability to inhibit NO production. Four non-polar fractions, F5, F6, F7 and F10 showed higher potency than the extract itself.

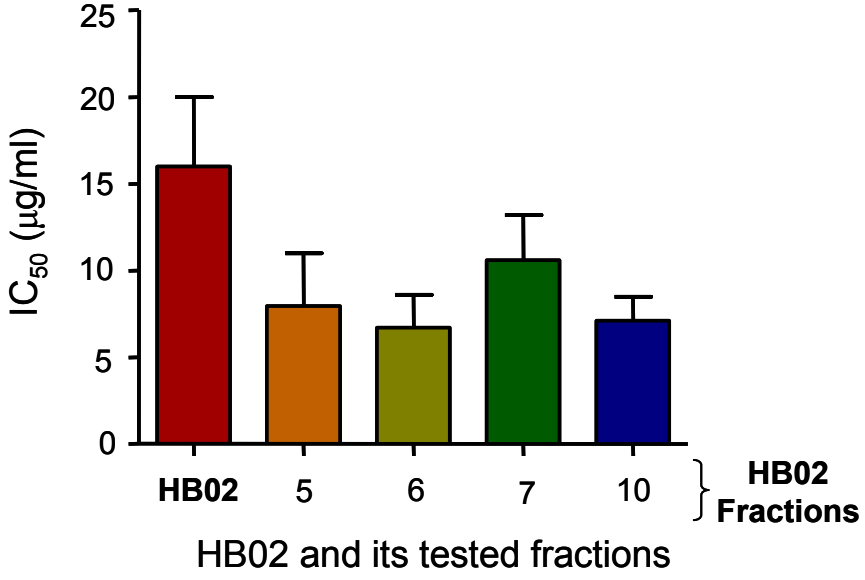


**Figure 4:** The median inhibitory concentration ($IC_{50}$) values of HBO2 extract from ZORT and its four fractions (F5, F6, F7, and F10) on NO production. Re-testing the extract and fractions showed that F6 had strongly inhibited NO production with the lowest $IC_{50}$ at 6.7±2.7µg/ml, more than 2-folds lower than HB02 at 16±5.7µg/ml.

## Discussion

The results confirmed that ginger possess potent anti-inflammatory effects comparable with widely-used synthetic pharmaceutical compounds. The results also provide insight into the best methods for the extraction potent anti-inflammatory effects of both plants. Chloroform is more efficient in extracting anti-inflammatory compounds from the ginger species. Chloroform is a non-polar solvent and, hence, extracts mostly non-polar compounds. In contrast, ethanol is a polar solvent and only dissolves polar compounds. In contrast, ethanol is a polar solvent and only dissolves polar compounds. In this respect the available evidence indicates that a majority of compounds with anti-inflammatory properties from plants are non-polar, such as phenypropanoids. This observation, therefore, indicates that the potent NO inhibitory effect observed for the chloroform extract, HB02 is likely to be due to the presence of non-polar anti-inflammatory compound(s) in the ginger species. The data also indicates that the inhibitory effects of HB02 and HC02 on NO production were comparable indicating that both ginger species most likely contain similar and, possibly, equal amounts of compounds with suppressive effects on NO production by macrophages. Thus, the results showed that in addition to suppressing NO and $PGE_2$ production that the nature of the constituent compounds could be identified. In this regard, fractionation of ZORT identified a number of fractions with anti-inflammatory effects amongst which F6 was the most potent. Assessment of F6, thus, showed it potently suppressed NO and PGE2 production at IC50 values lower that original extract from ZORT, or HB02 and also down regulated the iNOS at mRNA level as well as iNOS catalytic activity in NO production (data not shown). Detailed assessment of all 21 fractions obtained for HB02 also showed that in addition to F6, which had the most potent anti-inflammatory effects that F7 also displayed potent effects on reducing NO and PGE2 production. However, in contrast to F6, F7 was more effective in down regulating iNOS mRNA (not shown) when the macrophage cell line was treated after stimulation. Paradoxically, perhaps, F7 was more effective in reducing iNOS protein levels when the cells were treated with the fraction before LPS stimulation. This finding was in agreement with its effect on inhibition of NO production. The somewhat similar effects noted for F6 and F7 may be attributed to both fractions sharing similar, though not identical, components. However, the results also suggested that F7 (and F5) could have toxic effects on the cells. In contrast, F6 was not toxic, which supports the notion that although F6 and F7 have similar components, the both also have key differences in their composition Further studies of F6 showed that it was efficient in down-regulating iNOS levels both before and after the cells were stimulated and that F6 had lower IC50 than L-NAME, a specific inhibitor of NO used in these studies as a control. L-NAME is a L-arginine analogue and is a non-selective iNOS inhibitor 12. The compound acts by competing with L-arginine for binding to iNOS. Taken together, this implies that F6 inhibits NO production by, possibly, acting as an iNOS inhibitor. The inhibitory effects on the HB02 and F6 on both NO and $PGE_2$ production hints at possible links between the iNOS and COX-2 pathways. iNOS and its products have been shown to influence the COX pathway, particularly, COX-2 activation and PG production [13]. Among the PGs, $PGE_2$ is the most studied for its key role in inflammation. With this in mind, we studied the effect of HB02 and its fractions on $PGE_2$ production before and after LPS stimulation. The experiments showed that HB02 and the fractions had potent

inhibitory effects on $PGE_2$ production which were comparable to indomethacin (at 10µg/ml). Based on the proposed link between the iNOS and COX pathways, these results suggest that HB02 and the fractions affect both pathways. However, it remains to be determined whether either HB02 or the fractions'effects are specific or that they affect many enzymes amongst which are iNOS and/or COX1/2. There is also the possibility that inhibition of HB02 and its fractions on iNOS and COX1/2 could be through the transcription factor nuclear factor κB (NF-κB) activated through blocking the PI3K/Akt/IκB kinases IKK and MAPK [14]. To identify the key compounds with this potent anti-inflammatory action, experiments are underway to further fractionate F6. Preliminary studies suggest that compounds such as Shogaol and gingerdione (GD) could be involved in the anti-inflammatory effects of ginger.

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