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IN-VITRO IMMUNOMODULATORY AND CYTOTOXICITY POTENTIAL OF NATURAL COMPOUNDS FROM *FRAGARIA NUBICOLA*

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ABSTRACT: Introduction: Reactive oxygen species and nitric oxide play a major role in the physiopathology of inflammatory diseases, neurodegenerative disorders, autoimmune pathologies and immunodeficiency. With this understanding targeting oxidative stress could be a promising approach in new drug discovery. **Methodology:** Therefore, new compounds that were isolated from methanolic extract of leaves of wild strawberry, *Fragaria nubicola* were tested for its *in-vitro* anti-inflammatory activity against the free radical's generation during the process of respiratory burst by luminol enhanced chemiluminescence technique. The effect on superoxide and nitric oxide production was measured in cell culture supernatant by cell based spectrophotometric assay. The cytotoxicity analysis was done using a fibroblast cell line by MTT assay. **Results: a** current study has demonstrated the potential of selected compounds being an effective immunomodulating agent. These compounds (1-5) were found to be significantly inhibiting the myeloperoxidase dependent free radical generation with an IC_{50} value of 4.6 ± 1.3, 5.7 ± 1.6,<1, 2.3 ± 0.01 and <1 µg/mL respectively. As compare to ibuprofen, which showed IC_{50} of 43.4 ± 0.9 µg/mL. However, the compounds did not exert any effect on suppressing the myeloperoxidase independent pathway and not able to suppress superoxide anion. For nitric oxide production, the compounds 1 and 5 showed moderate activity with % inhibition of 35.1 and 38.3%, respectively. None of the compound showed cytotoxicity up to 25 µg/mL concentration. **Conclusion:** Results suggested that the new compounds were found to be selective inhibitors of myeloperoxidase dependent pathway of a respiratory burst with no cytotoxic effect. Revealing them as promising drugs, specifically targeting oxidative stress associated pathologies.

INTRODUCTION: During the process of inflammation, free radicals are generated by NADPH oxidase, a powerful oxidant- producing enzyme localized on the surface membrane of neutrophils or by inducible nitric oxide synthase (iNOS) expressed in activated phagocytic cells. ROS and RNS induce damage to macromolecules such as DNA, lipids, and proteins through their ability to induce biochemical alterations.

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Act as intracellular signaling molecules in many biological processes. For instance, increased concentrations of ROS/RNS are associated with the initiation of various pathologic conditions [1-5]. Therefore, one of the most promising inflammatory mediators is oxidative stress that is a state of imbalance between the body defense mechanism (anti-oxidant) and the free radicals generated during the process of immune activation [6].

Exploring natural compounds isolated from medicinal plants could be way to discover the novel therapeutic approaches to uncover the pathogenesis behind the inflammatory disorders. Targeting oxidative stress is one of the mechanistic approache for the treatment strategy of immune disorder [7].

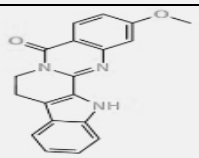
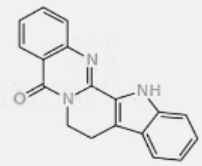
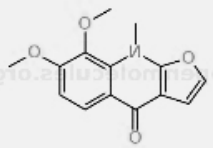
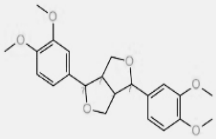
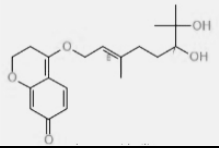
In this context, it is widely known that the leaves of Fragaria, a plant generally known as wild strawberry, has been utilized throughout the years by conventional drug for the treatment of a few sicknesses. In any case, logical reports revealing its molecular mechanism of activity are as yet deficient. The traditional uses of the plants, the isolation of their compounds, and the study of associated biological activities increased the medicinal value of the phytochemicals.

In general, there are 3 major groups of plants phytoconstituents terpenoids, alkaloids, and phenolic mixes [8-10]. Natural products are important sources of new drugs, and it has been widely shown that many plant-derived phytochemicals have considerable anti-inflammatory and anticancer effects [11]. Many plant polyphenols have anti-inflammatory properties, acting through different molecular mechanisms [12]. To identify the immune-modulating action of compounds *via*. Inhibition of free radicals produced during the inflammation, we have selected the 5 natural compounds previously isolated. The potential of natural compounds on intracellular oxidative stress using activated mouse macrophages and human peripheral mononuclear cells has been evaluated. The cytotoxic potential of the compounds has been done to rule out the toxic effect of natural compounds.

MATERIALS AND METHODS:

Collection of Compounds and Samples Preparation (1 mg/mL Stock Solution): 5 new compounds were isolated from fragaria nubicola. The structures of these compounds were elucidated by spectroscopic data given in **Table 1**. The stock solution of 1 mg/mL was prepared by adding 1 mg of compound in 50 µl DMSO mixed well, vortexed, and sonicatedtill completely solubilize. Finally, 950 µl PBS was added to achieve the final stock concentration of 1 mg/mL. After preparation labeled and stored in the freezer below 4 °C until use.

TABLE 1: SELECTED PURE COMPOUNDS AS MAJOR CONSTITUENT OF *FRAGARIA NUBICOLA*

S. no.	Structure	IUPAC Names	Compound Code
1		8,13-dihydro-2-methoxyindolo 2',3': 3,4]pyrido 2,1-b]quinazolin-5(7H)-one	Compound -1
2		8,13-dihydroindolo [2',3':3,4] pyrido [2,1-b]quinazolin-5(7H)-one	Compound -2
3		7,8-dimethoxy-9-methylfuro [2,3-b] quinolin-4(9H)-one	Compound -3
4		Hexahydro-1,4-bis(3,4-dimethoxyphenyl) furo [3,4-c] furane	Compound -4
5		4-((E)-6,7-dihydroxy-3,7-dimethyloct-2-enyloxy)-2H-chromene-7-one	Compound -5

Measurement of NO in Cell Culture Supernatant: The mouse macrophage cell line J774.2 (UK ECACC) was cultured in T 75 flasks in DMEM that contained 10% fetal bovine serum supplemented with streptomycin/penicillin 1%. Flasks were kept at 37 °C in atmosphere of humidified air containing 5% CO_2. J774 cells were collected and adjusted 10^6 cells per ml 200 µl of

these cells were dispense in 96 well microtiter plate and nitric oxide synthase (NOS-2) in macrophages was induced by Lipopolysaccharide from *E. coli* (30 µg/ml). The test compounds were added for initial screening at a concentration of 25 µg/ml, and for calculating (IC_{50}) compound was used at a concentration of 25, 5, and 0.5 µg/ml. After adding compounds, plates were incubated at 37 °C in 5% CO_2 Cell. Culture supernatant was collected after 24 h and immediately process for Nitrite accumulation test by Griess method, where 50 µl of 1% sulphanilamide in 2.5% phosphoric acid, followed by 50 µl of 0.1% naphtyl-ethylene diamine dihydrochloride in 2.5% phosphoric acid was added to 50 µl of culture medium. After 10 minutes of incubation at 23 °C the absorbance at 550 nm was read. Micro molar concentrations of nitrite were calculated from a standard curve constructed with sodium nitrite as a reference compound. Results were expressed as means ± SD of triplicate reading [13].

% Cell viability = Total viable cells (Unstained) / Total cells (Viable + Dead) × 100

Viable cells/ml = Average viable cell count per square × Dilution factor × 10

Determination for Oxidative Burst by Chemiluminescence Assay: Luminol enhanced chemiluminescence assay was performed as described earlier. In white half area 96 well plates Costar, NY, USA, the various concentration of compounds (1, 10 and 100 µg/mL) incubated at 37 ºC for 15 min in the thermostat chamber of luminometer Labsystems, Helsinki, Finland with whole blood (1:20 dilution in sterile HBSS ++). Control wells received HBSS ++ and cells but no compounds. After incubation, intracellular reactive oxygen detecting probe luminol working solution (7×10^{-5} M) and serum opsonized zymosan (SOZ) were added into each well except blank wells (containing only HBSS ++). The oxidative burst ROS production was monitored with the luminometer for 50 min in the repeated scan mode. The level of the ROS was recorded as total integral readings as relatively light units (RLU) [14].

Determination for Oxidative Burst by Superoxide Production NBT: Balb c mice were injected 1 ml FBS three days prior to sacrifice, each animal was killed by cervical dislocation, the mice were dipped in 100% ethanol, and 10 ml of RPMI-1640 medium, adjusted to pH 7.4 injected intra-peritoneal. The peritoneal exudate cells (PEC) was collected and centrifuge at 400 g for 10 min at 4 ºC, palette obtained was suspended in complete RPMI with 10% FBS. 100 µl of peritoneal macrophages (1 × 10 7) were seeded in 96 well cell culture plates in the RPMI 1640 media supplemented with 10% heat-inactivated FBS. Activity of NADPH oxidase was determined using a modified colorimetric nitro blue tetrazolium (NBT) assay as previously described. Cells were incubated with compounds for 30 min at 37 ºC, after incubation 100 µl of yellow-colored NBT solution (1 mg/ml) and 50 µl of phorbol-12- myristate-13-acetate (PMA) (10 µg/ml) was added and incubated again in same conditions for further, 90 min, for negative control diphenyleneiodonium was used. The blue color formazan formed can be extracted with organic solvent (DMSO), and the absorbance is read at 570 nm. Control was run simultaneously using cells in medium instead of compounds [15]. The percentage inhibition of NBT reduction was calculated by.

% Inhibition = 100 - (OD of sample treated cells - OD of cells alone) × 100 / (OD of PMA treated cells - OD of cells alone)

***In-vitro* Cytotoxicity Assay:** The cell growth inhibition effect of compounds was determined by using the MTT assay, 3T3 NIH (Mouse fibroblast), grown in MEM (Modified Eagle's medium) containing 10% FBS and 1% Antibiotic (Penicillin and streptomycin), 1% glutamine and maintained at 37 ºC with 5% CO_2 level for 72 h in the flask. Cells 6×10^4 of 3T3 were placed in 96 well flat-bottom plates for 24 h to allow the cells attachment. Next day media was replaced by fresh media, and various concentrations of samples 25, 5, 0.5 µg/mL were added into the well and further incubated for 48 h. After that medium was again removed by flipping the plate MTT was added 2 ml of MTT (3-(4, 5 - Dimethylthiazol - 2 - yl) - 2, 5 - diphenyl tetrazolium bromide) (5 mg/mL) in 18 ml of medium loaded as 200 µL to the wells, after 4 hours Medium and reagents were aspirated and 100 µL DMSO was added and mixed thoroughly for 15 minutes to dissolve the formazan crystals. The absorbance was measured at 570 nm using microplate spectra max 340 (Molecular Devices, CA, USA). In the end, IC_{50} values were calculated, and at least three independent experiments were

carried out for each sample. Cycloheximide was used in this assay, as a positive control [16].

Statistics: The values are expressed as means ± SD. The significance of difference from the respective. Controls for each experimental test condition were assayed by using one-way ANOVA for each paired experiment. P-value 0.05 was regarded as indicating significant differences. Denoted as * $p<0.05$ and $p< 0.005$ as **

RESULTS:

Effect of Natural Compounds on Nitric Oxide: LPS treated J774.2 mouse macrophage cell line has been widely used to study the mechanisms of nitric oxide synthase (NOS-2) induction. Here we studied the effect of natural compounds on nitric oxide production by activated macrophages, and we found that out of all 5 compounds, 1 and 5 suppressed the nitric oxide moderately with a percentage of inhibition of 35.1 and 38.3% at 25 µg/mL concentrations respectively. Whereas compound 2, 3 and 4 showed little nitric oxide inhibition with % inhibition of 26.6, 27.6, 29.3%, respectively **Table 2**.

Effect of Natural Compounds on Reactive Oxygen Species: Luminol enhanced chemilluminessence technique mainly detects the HOCl, hypochlorous acid produced during the process of phagocytosis that is myeloperoxidase-dependent intracellular killing process**.** The peripheral whole blood cells were activated with zymosan in the presence of compounds. Results showed signify-cantinhibitory activity $p<0.005$ against reactive oxygen species (ROS) production. All compounds were found to exert potential inhibition on ROS generation. Compound 3 and 5 showed IC_{50} values of even less than 1 µg/mL indicating their suppressive effect on ROS and compound 1, 2 and 4 showed IC_{50} values of 4.6 ± 1.3, 5.7 ± 1.6 and 2.3 ± 0.0 µg/mL respectively. As compare to ibuprofen, which showed IC_{50} of 43.4 ± 0.9 µg/mL **Table 2**.

Effect of Natural Compounds on Superoxide Production: Compounds were incubated in PMA activated mouse macrophages at concentration of 25 µg/mL to find the effect on PMA induced superoxide release by mouse macrophages in NBT reduction assay for superoxide that mainly detected the superoxide production during myeloperoxidase-independent intracellular killing process. All were found to be inactive except compound 2 showed little inhibitory activity of 22% inhibition **Table 2**.

TABLE 2: EFFECT OF NATURAL COMPOUNDS ON ROS, NO AND O^{-2} PRODUCTION IN ACTIVATED MACROPHAGES AND NEUTROPHILS AND CYTOTOXICITY ANALYSIS

Compounds	ROS IC_{50} ± SDV µg/mL	Superoxide O^{-2} % Inhibition ± SDV	Nitric oxide (NO) % Inhibition ± SDV	Cytotoxicity IC_{50} ± SDV µg/mL
Compounds	4.6 ± 1.3**	9.0 ± 0.21	35.1 ± 0.12	>25
Compounds	5.7 ± 1.6**	22.6 ± 0.30	26.6 ± 0.66	>25
Compounds	<1**	9.9 ± 0.41	27.6 ± 0.80	>25
Compounds	2.3 ± 0.0**	33.4 ± 0.8	29.3 ± 0.26	>25
Compounds	<1**	17.1 ± 0.77	38.3 ± 0.24	>25
Standard drugs	Ibuprofen 43.4 ± 0.9 ug/mL	Diphenyl iodonium 92 ± 0.94%	N^G monomethyl L arginine 64 ± 0.05%	Cyclohexamide 0.13 ± 0.02µg/mL

ROS produced by human blood phagocytes and NO and O^{-2} produced by mouse macrophages were determined as described in material and methods. Results are presented as means ± SD of triplicate measurements. The cytotoxic assay was done using MTT Assay on 3t3 fibroblast cells **$p<0.005$

Effect of Natural Compound on Cytotoxicity: Effect of compounds on cell viability was determined on 3T3 Fibroblast cells; the effect on cellular toxicity was compared with the standard drug Cycloheximide.

The data is presented in **Table 2**. The compounds were added at a final concentration of 50, 5, and 0.5 µg/mL, to identify the IC_{50} value. None of the compound showed any toxicity until 50 µg/mL on fibroblast cells. Compound found to be safe and not toxic with IC_{50}value of > 25 µg/mL as compared to the Cycloheximide, which is inhibiting the cell growth upto 71% with IC_{50} value of 0.13 ± 0.07 µg/Ml.

DISCUSSION: Overproduction of NO can cause the production of a very toxic radical peroxynitrite and may reduce the bioavailability of physiological NO necessary for neurotransmission as well as

vasodilation; therefore the suppression of NO could be a lead to control the heart-related disorders for instance hypertension and Atherosclerosis [17]. In the current study, two compounds 1 and 5 moderately inhibited the nitric oxide released in the supernatant and could be of clinical importance if further explored. On the contrary, all the studied compounds have a significant effect on Myeloperoxidase-dependent intracellular release of reactive oxygen species. Myeloperoxidase utilizes H_2O_2 and halide ions (usually Cl^-) to produce highly toxic hypochlorite. Some hypochlorite spontaneously breaks down to yield singlet oxygen. Together these reactions produce toxic hypochlorite (OCl^-) and ($^1O_2$) singlet oxygen [18]. All the compounds being inhibiting the reactive oxygen species detected by luminol enhance chemoluminescence assay suggested that the compound is a specific inhibitor of this pathway.

On the other hand, during phagocytosis the process of myeloperoxidase-independent intracellular killing activates NADPH oxidase, which utilizes oxygen to oxidize the NADPH results in the production of superoxide anion. These superoxide anions can react with H_2O_2, resulting in the formation of hydroxyl radical plus more singlet oxygen and (O^{2-}) superoxide anion [19]. Our compounds showed no activity against superoxide production; that is why they are in effect against the NADPH oxidase enzyme or myeloperoxidase independent pathway.

However, these compounds are a specific inhibitor of myeloperoxidase dependent intracellular reactive oxygen release that may ensure the specific target against inflammation. Free radicals generated during this inflammation are harmful to the cell, and progressive release of these radicals lead to inflammation [20-22] and establish the inflammatory disorders. The current study could lead to the discovery of target-specific oxidative stress inhibitors. The identification of *in-vitro* cytotoxicity of compounds is significant to elucidate the effectiveness of natural compounds since most of the natural compounds possess the potential toxicity. The compounds were tested for its toxicity effect on the fibroblast cell line, and interestingly, none of the compounds was toxic to fibroblast cells until 50 µg/mL, as compared to Cycloheximide. Cyclo-hexamide is widely used in biomedical research to inhibit protein synthesis in normal eukaryotic cells [23]. Therefore, we use the compound as our positive control in the experiment to compare the toxic effect of new compounds from *Fragaria nubicola*.

CONCLUSION: Effect of natural compounds isolated from *Fragaria nubicola* showed a significant suppression on myeloperoxidase dependent respiratory burst; this effect could be due to inhibition of enzymes involve in hypochlorite anion generation. In addition, the compounds showed little to moderate effect on other reactive species, including superoxide and nitric oxide that may involve NADPH Oxidase and iNOS (inducible nitric oxide synthase), indicating that compounds are not a nonspecific inhibitor.

Cytotoxicity effect of compounds revealed that these compounds possess the potential of being safe compounds and as well cytoprotective via suppressing the free radical generation at cellular level effectively and specifically. Therefore, these compounds have the potential of the anti-inflammatory drug; however, further analysis is needed for a better understanding of the exact mechanisms of its action.

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