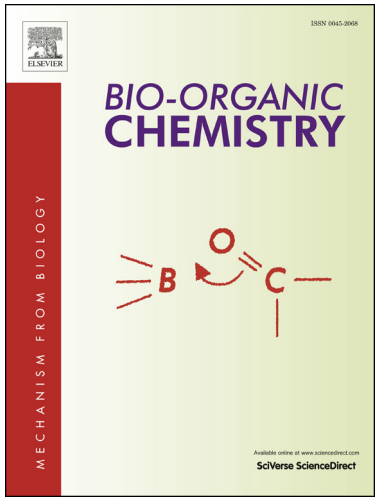

Synthetic indole Mannich bases: Their ability to modulate *in vitro* cellular immunity

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Synthetic indole Mannich bases: Their ability to modulate *in vitro* cellular immunity

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ABSTRACT:

The synthetic indole Mannich bases **1**-**13** have been investigated for their ability to modulate immune responses measured *in vitro*. These activities were based on monitoring their affects on T-lymphocyte proliferation, reactive oxygen species (ROS), IL (interleukin)-2, IL-4, and nitric oxide production. Compound **5** was found to be the most potent immunomodulator in this context. Four of the synthesized compounds, **5**, **11**, **12**, and **13**, have significant potent inhibitory effects on T-cell proliferation, IL-4, and nitric oxide production. However, none of the thirteen indole compounds exerted any activity against ROS production.

Keywords: Indole Mannich base, ROS, Nitric Oxide, Cytokine Inhibition.

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1. Introduction

The indole moiety is a repeating scaffold in the kingdom of natural products [1]. Several medicinally-applied indole derivatives showed antitumor activity [2], and also can lead to vessication and inflammation of human skin [3]. Several indoles derivatives like thiopyrano-indoles have analgesic effects [4], various indole alkaloids like aspidospermine and yohimbine possess wide range of bioactivity profiles [5]. The indole compounds have 5-HT receptor activity as agonists [6] or antagonists [7].

Indoles derivatives have been referred to as fortunate structures since it have the ability to bind with many receptors [8,9]. The indole derivatives substituted at 3-position are of great intrest due to their various biological activities [10-13].

β- and *γ*-Carbolines, having indole moiety are the significant structural units for various biologically important alkaloids [14,15]. They have potent anticancer, CNS [16] and antitumor activity [17]. The ellipticine and olivacine are most important anticancer agents.

The indole alkaloids of the *Ergot* family are of great importance [18], the most valuable representative of this family is lysergic acid [19-21]. Nevertheless, other members of this family have also played important roles in natural product chemistry. Some of these alkaloids such as setoclavine [22] and rugulovasines showed potent dopamine agonist activity and reiterates the importance of the indole moiety in natural products [23].

T-cell proliferation and oxidative burst activities are two indicators for compounds influencing cellular and innate immune response. IL-4 is an important immunomodulatory cytokine expressed by a highly restricted pattern of cells of distinct lineages located in diverse locations in the body such as circulating T-cells. IL-4 plays an essential role in protective immunity as well as in inflammatory diseases, making IL-4 an attractive target for inhibition to protect the body from inflammatory disorders. NO is a free radical molecule which is produced in large quantities during inflammatory reaction and due to its cytotoxic properties to normal cells; its signaling is involved in many physiological and pathological processes [24-31].

Numerous classes of compounds have been reported as immunomodulators. Fukahori *et.al.* reported recently the effect of AS2521780, a potent PKCθ inhibitor, on T cell-mediated immunity. It also suppressed CD3/CD28-induced Interleukin-2 gene transcription in Jurkat T cells and proliferation of human primary T cells [32]. Acanthocheilonema viteae product ES-62 a small synthetic molecule have been reported to prevents development of collagen-induced arthritis [33]. The diterpenoids polyandric acid A (1), has been checked for inhibition of pro-inflammatory cytokine production and other inflammatory mediators and was found to be significantly inhibited interleukin-1β production [34]. New sesquiterpenoids, and monoterpenoid were isolated from the rhizomes of Curcuma wenyujin and found to be strongly inhibited the induction of NO production by LPS [35]. In the context to design and discover new immunomodulators, thirteen Mannich bases were examined for their effects on T-cell proliferation, IL-2 and IL-4 production of peripheral blood mononuclear cells (PBMCs), reactive oxygen species (ROS) production on whole blood phagocytes, and nitric oxide production of mouse macrophage cell line J774.2.

2. Results and Discussion

2.1.Chemistry

In order to extend our research: on bioactive compounds [36]. Herein we are going to report synthesis of indole Mannich bases as fellow. Indole (3 mmol), formaldehyde (1.2 mmol), secondary amine (3 mmol), and distilled water (15 ml) were added to a reaction flask and the solution was refluxed for 2 h with vigorous stirring. Completion of the reaction was periodically monitored by TLC. In most cases the resulting solid mixture was collected on a filter. If no precipitate formed, the Mannich bases were extracted with ethyl acetate, purified by column chromatography using a mixture of acetone/hexane (1:1, v/v) as eluent, yielding pure products 1-13 in yields ranging from 75-89% (Scheme-1) [37].

Insert Scheme-1 Here

2.2.Bioactivities

2.2.1. Effect of compounds on PHA-activated T-cell proliferation

T-cells undergo a clonal proliferation, when stimulated *in vitro* by mitogens such as phytohemagglutinin (PHA-P), concanavalin A (Con A) or pokeweed mitogen (PWM). The level of proliferation is determined by adding radioactive ^{3}H thymidine to the cells merging into the freshly synthesized DNA of the dividing cells. The amount of radioactivity as determined in a scintillation counter is related to the number of newly produced cells. In the current study thirteen synthetic Mannich bases were screened for their effect on cellular immune responses. T-Cell proliferation was initiated using the PHA-P mitogen to activate the PBMCs, isolated from healthy human volunteers. Initial screening was performed using a single dose (10 μg/mL) of each compound, and those that inhibited cell proliferation by 50% or more were further analyzed for their IC_{50} activity (Table-2). Out of thirteen compounds only four, **5**, **11**, **12**, and **13** were found to inhibit T-cell proliferation with significant inhibitory activities with IC_{50} value of 1.6, 2.0, 1.6, and 3.5 μg/mL, respectively (Table-1, Figure-1).

Insert Table-1 Here

Insert Figure-1 Here

2.2.2. Effect of compounds on Nitrite production:

Nitrous oxide (NO) is a free radical molecule produced in large quantities during inflammation and due to its cytotoxic properties to normal cells its signaling is involved in many physiological and pathological processes [38]. Therefore, our indole Mannich bases were investigated for their potential inhibitory effect on nitrite generation using stimulated murine macrophages cells (J774.2). In this study, LPS-treated J774.2 cells were used for the nitrite production *via* induction of nitric oxide synthase (NOS). Among the studied compounds **1**, **9**, and **11** had moderate inhibitory activity with a percentage inhibition from 40% to 60%, whereas, compound**s** **5**, **12**, and **13** showed highly significant ($P \leq 0.005$) inhibitory effects on nitrite production with a percentage of inhibition > 90 % at a dose of 25 μg/mL (Table-2).

Insert Table-2 Here

2.2.3. Effect of Mannich Bases 5, 11, 12, and 13 on ROS production in human whole blood

Phagocytes upon activation by zymosan or phorbol 12-myristate 13-acetate (PMA) release reactive oxygen species (ROS) quantifiable by a luminol-enhanced chemiluminescence assay [38]. Compounds **1**-**13** (Table-2) were screened for production of ROS using whole blood and luminol for ROS detection. While compound **4** had a moderate inhibitory activity (IC_{50} 27.1 μg/mL), compounds **3, 11**, and **12** showed effects with IC_{50} values ranging between 41.9-47.8 μg/mL. The rest of the studied compounds did not show any significant activity up to the highest concentration (100 μg/mL) tested (Figure-2).

Insert Figure-2 Here

2.2.4. Effect of Mannich Bases 5, 11, 12, and 13 on IL-4 production of peripheral blood mononuclear cells

Production of IL-4 which is of critical importance for Th2 cell activation and differentiation, was also studied for the four Mannich bases having significant inhibitory effect against T-cell proliferation, and, indeed, compounds **5**, **12**, and **13** suppressed IL-4 production significantly with IC_{50} values of 5.5, 6.3, and 8.4 μg/mL, respectively (Figure-3).

Insert Figure-3 Here

2.2.5. Effect of compounds on IL-2 production:

To evaluate the effect of indole Mannich bases **5**, **11**, **12**, and **13** (inhibiting T-cell proliferation, see above) on the production of interleukin 2 (IL-2) by Jurkat T-cells, we found that only two compounds, **5** and **12**, have moderate IL-2 inhibitory activity with IC_{50} values of 11.8 and 13 μg/mL (Figure-4).

Insert Figure-4 Here

2.2.6. Cytotoxicity of compounds 5, 11, 12, and13

Cytotoxicity of compounds **5, 11, 12**, and**13** was tested on the NIH 3T3 mouse embryonic fibroblast cells at concentrations of 1, 5 and 25 μg/mL which showed viability between 80-100 % at the highest concentration tested (25 μg/mL) except compound **12** with an IC_{50} value f 12.5 μg/mL.

2.2.7. Statistical analysis

All data are reported as mean ± SD of the mean and the student t-test was used to determine the difference between test- and control preparations significance was attributed to probability values P 6 0.05. The IC_{50} values were calculated using Excel based program.

3. Experimental

NMR experiments were performed on Avance Bruker AM 300 MHz. CHN analysis was done on a Carlo Erba Strumentazion-Mod-1106, Italy. Electron impact mass spectra (EI MS) were recorded on a Finnigan MAT-311A, Germany. Thin layer chromatography was accomplished on pre-coated silica gel aluminum plates (Kieselgel 60, 254, E.Merck, Germany). Chromatograms were visualized by UV at 254 and 365 nm. Ultraviolet (UV) spectra were recorded on Perkin-Elmer Lambda-5 UV/VIS spectrometer in MeOH. Infrared (IR) spectra were recorded on JASCO IR-A-302 spectrometer as KBr (disc).

*3.1.*T-Cell proliferation assay

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood of healthy adult donor by Ficoll-Hypaque gradient centrifugation [39]. Cells were proliferated following a method reported by Nielsen *et al* [40]. Briefly cells were cultured at a concentration of 5 · 105/mL in a 96 well round bottom tissue culture plate (Nalge Nunc. Inter.). Cells were stimulated with 1.25 μg/mL of phytohemagglutinin (Sigma Co.,USA). Various concentrations of oxazolone compounds were added to give final concentrations of 7.5, 16, 25 and 50 μg/mL each in triplicate. The plate was incubated for 72 h at 37 °C in 5% CO_2 incubator. After 72 h, cells were pulsed with (0.5 μCi/well)

tritiated thymidine, and further incubated for 18 h. Cells were harvested onto a glass fiber filter (Cambridge Technology, USA) using cell harvester (SKATRON A.S. Flow Lab. Norway). The tritiated thymidine incorporation into the cells was measured by a liquid scintillation counter (1211 LKB WALLAC). CPM results were recorded after 120 s.

3.2. Cytokine production from mononuclear cells

Cytokine from mononuclear cells was assayed using the human cytokine kits (Diaclone, Besancon Cedex,France). Briefly, freshly prepared mononuclear cells (105/well) were cultured in 96-well microtiter plate in the presence or absence of 1.25 g/mL PHA. Four different concentrations (0.5, 1, 4 and 16 μg/mL) of each compound along with PHA were used in this assay. Plate was incubated at 37 ^{o}C in CO_2 environment. After 18 h of incubation, the supernatant was collected and analyzed for IL-2, IL-4 and IFN-γ cytokine production.

3.3. Cytotoxicity evaluation

The MTT test was performed to evaluate the cytotoxic effect of the compounds according to the following method described previously [41]. Briefly MTT was dissolved in 1 · PBS at 2 mg/mL. Mavin Darby bovine kidney (MDBK) adherent cell (2 · 105) were incubated with serial concentrations of compounds (0.195–50 lg/mL) for three/five days. Supernatant was removed and 50 lL of MTT solution was then added. Plates were incubated for an additional 2-3 h at 37 ^{o}C in a tissue culture incubator. MTT was aspirated off and 200 lL of DMSO was added to dissolve the formazan crystal
formed by living cells. The plate was agitated at room temperature for 15 min then read at 540 nm using microplate reader.

3.4. Procedure

Indole (3 mmol), formaldehyde (1.2 mmol), and secondary amine (3 mmol), ml were mixed in reaction flask and then add distill water 10-15 and refluxed the solution for 2 h with vigorous stirring. The reaction completion was checked by TLC, and the resulting solid mixture was then filter. In some cases solid mixture was not formed so it was extracted with ethyl acetate, purified by column chromatography using acetone and hexane system in 1:1 ratio, and pure product is obtained in 75-89 % yield [37].

3.4.1. *3-((4-phenylpiperazin-1-yl)methyl)-1H-indole* (**1**)

3.4.2. 3-((4-phenylpiperazin-1-yl)methyl)-1H-indole-5-carbonitrile (2)

3.4.3. 3-((4-methylpiperazin-1-yl)methyl)-1H-indole-5-carbonitrile (3)

3.4.4. 5-bromo-3-((4-ethylpiperazin-1-yl)methyl)-1H-indole (4)

Representative compound UV, IR, CHN, EI-MS and ^{1}H-NMR data

3.4.5. 2-(1-{4-[(5-Bromo-1H-indol-3-yl)methyl]-1-piperazinyl}vinyl)-2,4-cyclopentadien-1-ol **(5)**

Rf: 0.51 (ethyl acetate/hexane, 3:7); UV (MeOH) λmax 283 (log ε = 4.2) nm; IR (KBr): νmax 3062, 2922, 1603 cm-1; 1H-NMR (300 MHz, CDCl3): δ 10.31 (s, 1H, NH), 7.90 (d, 1H, $J_{4,6}$ = 1.8 H-4), 7.64 (dd, 1H, $J_{4',2'}$ = 0.6, $J_{4',3'}$ = 2.4 Hz, H-4'), 7.37 (d, 1H, $J_{7,6}$ = 8.7 Hz, H-7), 7.32 (d, 1H, J_2,CH2 = 2.1, H-2), 7.21 (dd, $J_{6,4}$ = 2.1, $J_{6,7}$ = 8.7 Hz, H-6), 6.91 (dd, 1H, $J_{2',4'}$ = 0.6, $J_{2',3'}$ = 3.3 Hz, H-2'), 6.53 (q, 1H, $J_{3',2',5'}$=1.8 Hz, H-4'), 3.70 (s, 6H, Aryl-CH_2/ H-3",5"), 2.49 (br.s, H-2",6"); MS: *m/z* (rel. abund. %), 387 (M+, 30), 193 (93), 138 (62), 95 (100). Anal. calcd. For C18H18BrN3O2 (387.06); C, 55.68; H, 4.67; Br, 20.58; N, 10.82; O, 8.24 Found C, C, 55.67; H, 4.65; Br, 20.56; N, 10.80; O, 8.23

3.4.6. 3-((4-(furan-2-carbonyl)piperazin-1-yl)methyl)-1H-indole-5-carbonitrile (6)

3.4.7. 3-((4-(4-methoxyphenyl)piperazin-1-yl)methyl)-1H-indole-5-carbonitrile (7)

3.4.8. 3-((4-acetylpiperazin-1-yl)methyl)-1H-indole-5-carbonitrile (8)

3.4.9. (4-((1H-indol-3-yl)methyl)piperazin-1-yl)(furan-2-yl)methanone (9

3.4.10. 5-bromo-3-((4-phenylpiperazin-1-yl)methyl)-1H-indole) (10)

3.4.11. 3-((4-(4-methoxyphenyl)piperazin-1-yl)methyl)-1H-indole (11)

3.4.12. 5-methoxy-3-((4-(2-methoxyphenyl)piperazin-1-yl)methyl)-1H-indole (12)

3.4.13. ethyl 4-((5-methoxy-1H-indol-3-yl)methyl)piperazine-1-carboxylate (13)

4.0. Conclusion

The presented studies show that four of the new indole Mannich bases (**5**, **11**, **12**, and **13**) have significant potent inhibitory effects on T-cell proliferation, IL-4 and nitric oxide production. Compounds **5** and **12** exert moderate inhibitory effects against IL-2 production, while none of the thirteen indole compounds showed activity against ROS

production. Therefore, these active compounds need to be further investigated for the development of anti-inflammatory lead compounds. The new immunoactive Mannich base may serve as lead structures for the development of anti-inflammatory remedies with increased specificity and less side effects.

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Captions

Scheme-1: Synthesis of indole Mannich bases.

Table 1: Inhibition (%) and IC_{50} values of T-cell proliferation at 10 μg/mL, IC_{50} of IL-4 and cytotoxicity results for indole Mannich bases. (-) sign means IC_{50} not done because inhibition (%) of the compound is < 50.

Figure 1: Effect of indole Mannich basees **5**, **11**, **12**, and **13** on the proliferation of phytohemagglutinin (PHA)-activated T-cells. T-Cell proliferation was initiated using the PHA-P

mitogen to activate the PBMCs, compounds were added (50, 5, and 0.5 μg/mL) to check their effect on proliferation. Level of proliferation was determined by adding radioactive ^{3}H thymidine. Each bar represents the mean value of triplicate reading ± SD.

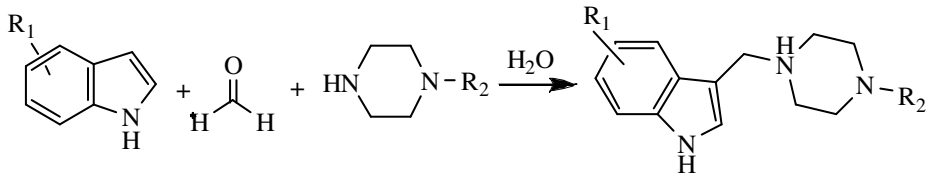
Table-2: Effect of compounds **1-13** on nitrite production by mouse macrophages J774.2. Macrophages were incubated in presence or absence of 25 μg/mL of compounds for 48 h. The supernatant was collected to detect the presence of nitrite by Griess reagent. (N^{G}-monomethyl-L-arginine (LNMA) is a known inhibitor of NO.

Figure 2: Effect of indole Mannich bases **1-13** on oxidative burst, ROS production using whole blood phagocytes. Zymosan activated whole blood cells were incubated in presence or absence of compounds (100, 10, and 1 μg/mL) for 30 min. Luminol was added at the end of reaction and relative light units were measured using chemilluminiscence. Each bar represents the mean value of triplicate reading ± SD.

Figure 3: Effect of indole Mannich bases **5**, **11**, **12**, and **13** on the production of IL-4 from human peripheral blood mononuclear cells (PBMCs). PMA activated T cells were incubated with compounds (25, 5, and 1 μg/mL) for 48 h. The supernatant was collected and analyzed for IL-4. + C indicates PMA activated cells without compounds. - C indicates base line IL-4 level. Each bar represents the mean value of triplicate reading ± SD.

Figure-4: Effect of indole Mannich bases **5**, **11**, **12**, and **13** on the production of IL-2 by Jurkat cells. PMA activated T cells were incubated with compounds (2, 5, and 0.5 μg/mL) for 48 h. The supernatant was collected to detect the IL-2 using R&D kit. +C indicates activated cells**.** Each bar represents the mean value of triplicate reading ± SD.

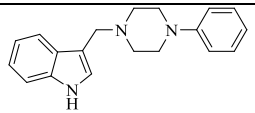
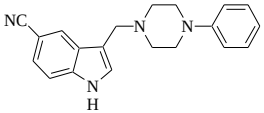
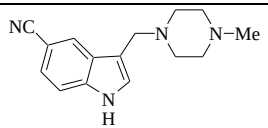
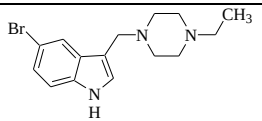
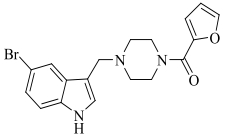
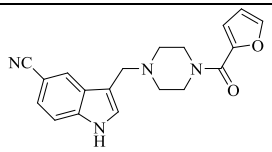
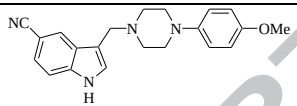
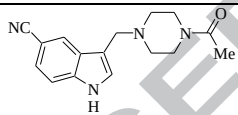
Scheme-1:



R_1 = CN, H, Br, OMe,

R_2 = alkyl or Aryl

Table-1

Compounds	Structures of Compounds	% of T-cell Inhibition (10 μg/mL)	T-Cell IC_{50} μg/mL	IL-4 inhibition (mg/ mL)	
				IL-4 inhibition at 10 mg/mL	IC_{50}
1		44.2	-	-	-
2		11.2	-	-	-
3		18.4	-	-	-
4		12.5	-	-	-
5		**98.4**	1.6	**93.9**	**5.0 ± 1.8**
6		14.3	-	-	-
7		3.3	-	-	-
8		17.9	-	-	-

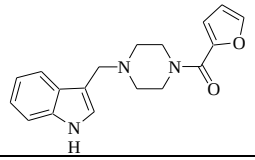
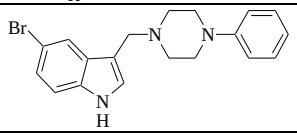
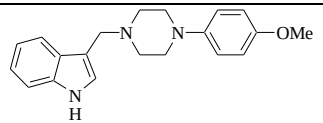
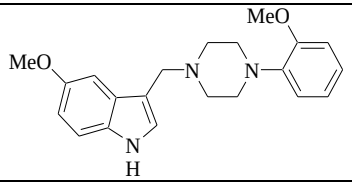
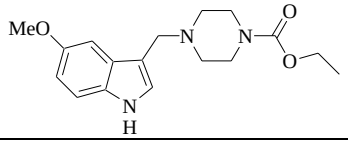
9		30.9	-	-	-
10		20.1	-	-	-
11		**99.6**	2.0	**82.9**	**8.4 ± 3.8**
12		**96.4**	1.6	**86.6**	**6.3 ± 0.5**
13		**83.0**	3.5	**64.9**	**10.7 ± 3.2**

Table-2:

Compound No.	% inhibition of NO
1	47.8
2	15.9
3	5.8
4	16.9
5	**97.0**
6	14.3
7	9.0
8	9.2
9	**47.6**
10	11.6
11	**68.3**
12	**96.9**
13	**92.2**
LNMA	**64.5**

Figure-1:

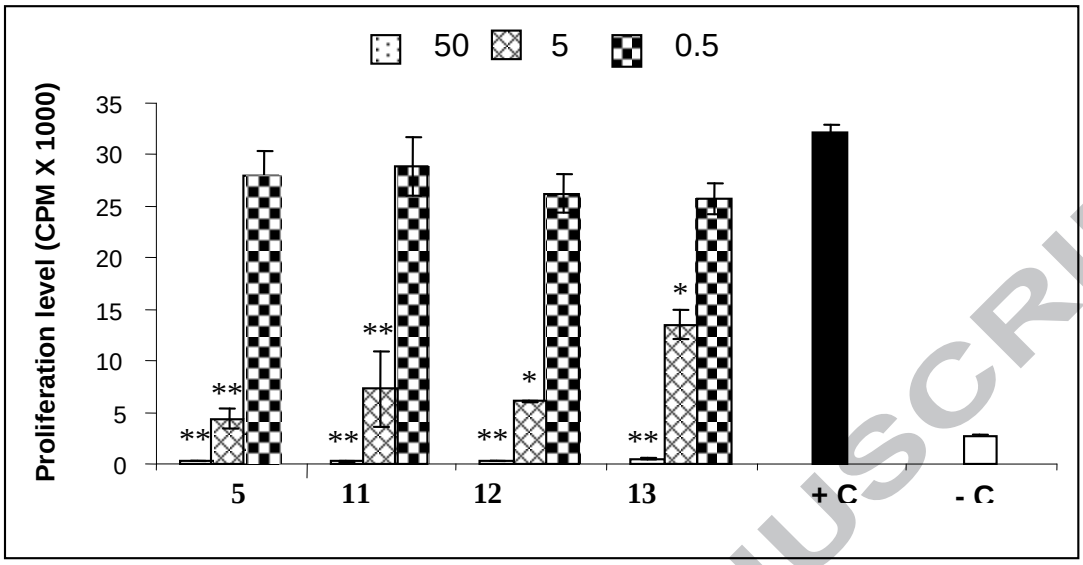


Figure-2:

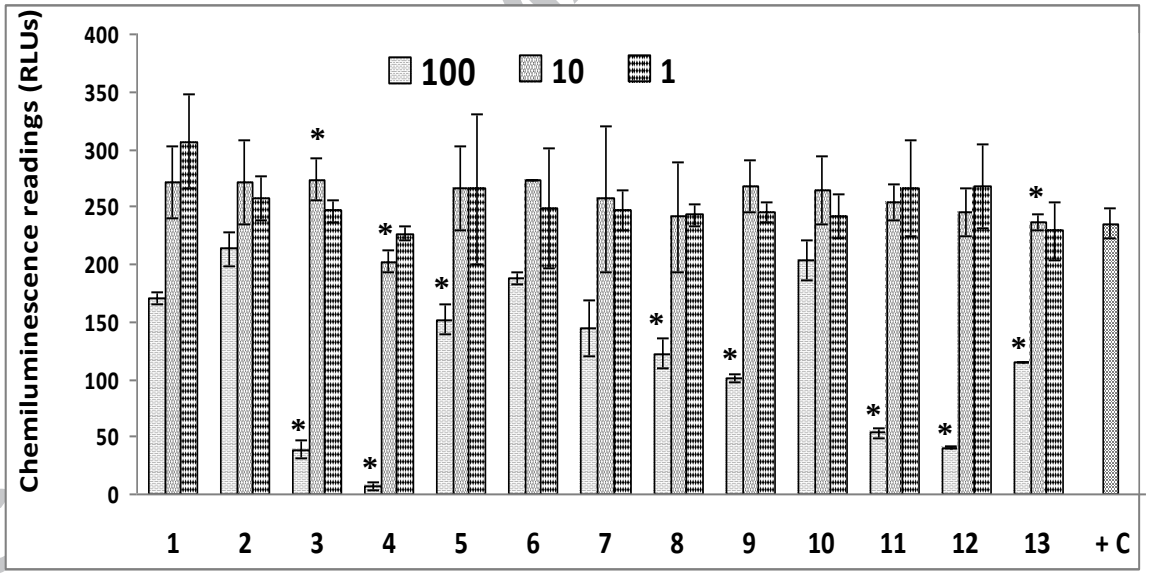


Figure-3:

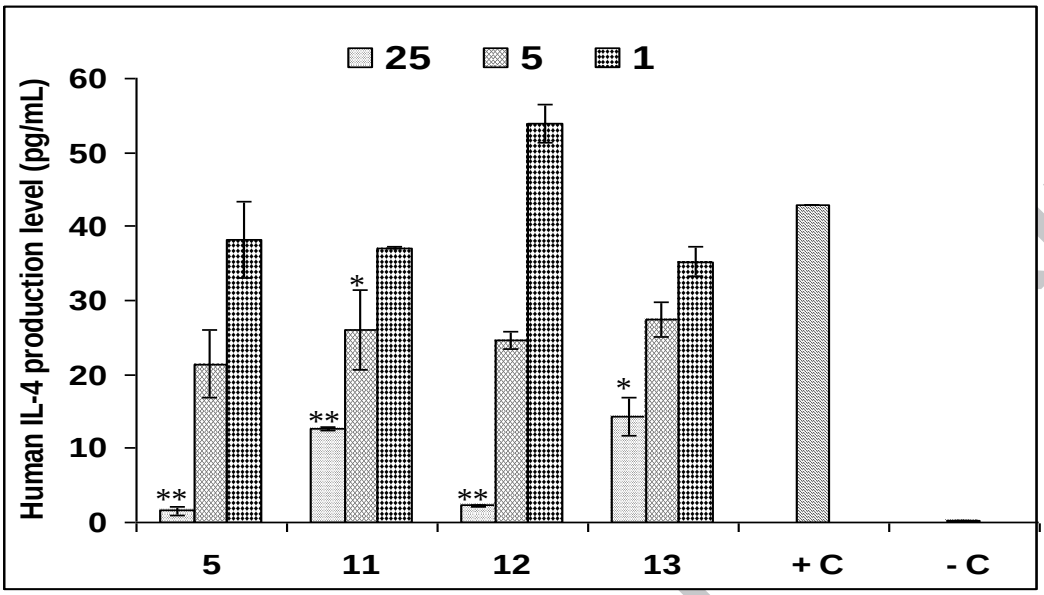


Figure-4:

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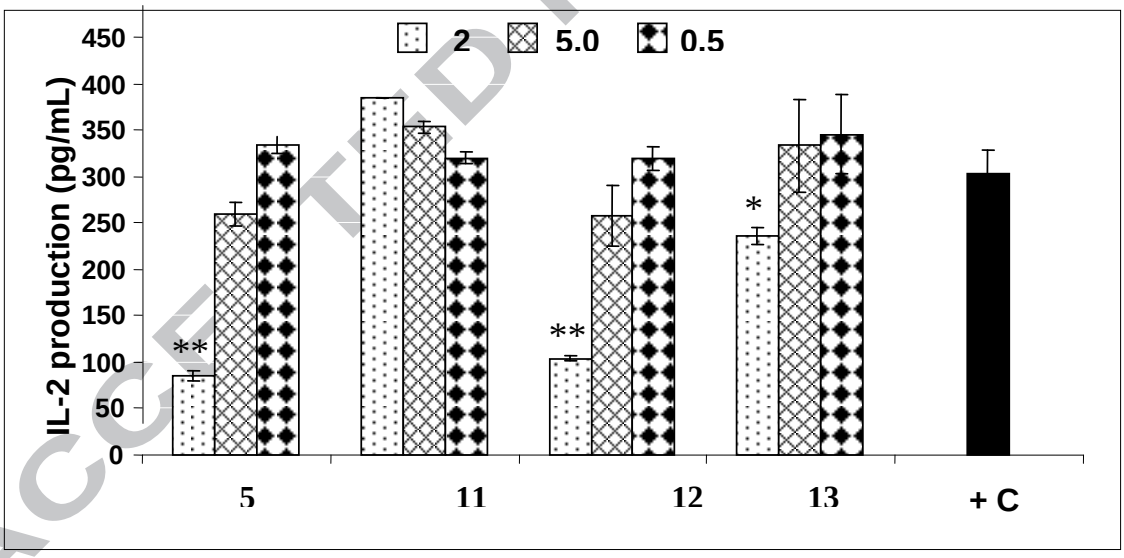

Synthetic indole Mannich bases: Their ability to modulate *in vitro* cellular immunity

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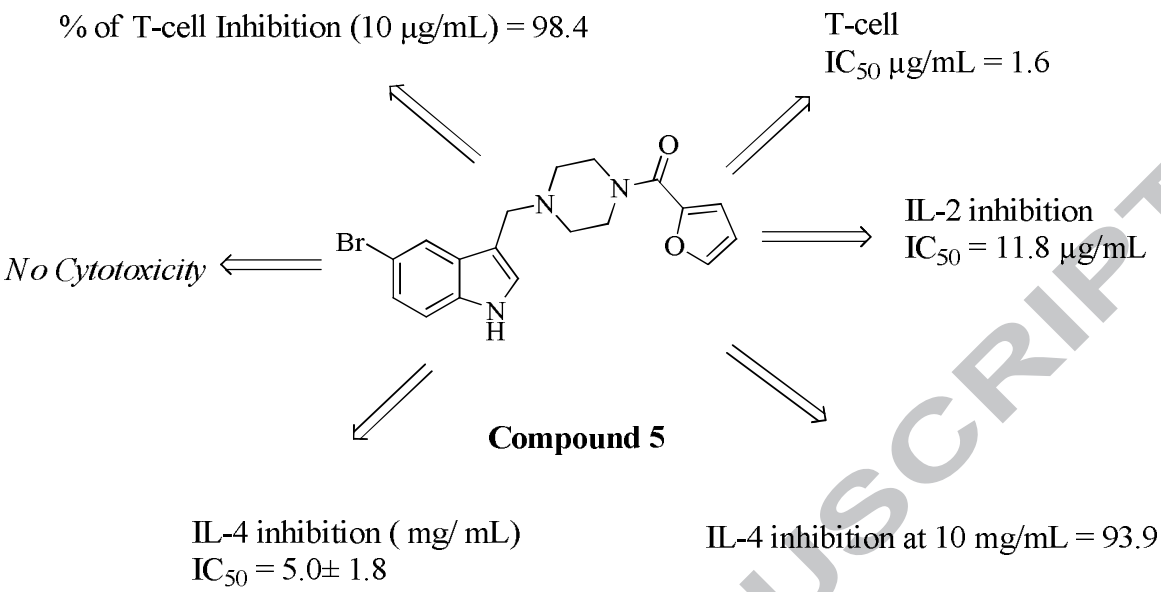

HIGHLIGHTS

- Indole Mannich base derivatives
- ROS
- Nitic Oxide
- Cytokine Inhibition