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Synthesis and evaluation of some novel derivatives of 2-propoxybenzylideneisonicotinohydrazide for their potential antimicrobial activity

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Abstract: A novel series of Mannich bases containing isoniazid were prepared. First, reaction of 2-propoxybenzaldehyde with isoniazid gave the corresponding hydrazone (**2a**). Subsequently, product **2a** after the Mannich reaction of aminomethylation with formaldehyde and secondary amines gave **2b**–**k**. The inhibitory potencies of the synthesized compounds were assayed *in vitro* against a panel of microorganisms and against the A549 human lung adenocarcinoma cell line. Compounds **2c** and **2k** displayed moderate to potent antimicrobial activity against all the tested strains and they also exhibited significant cytotoxicity in a dose-dependent manner with IC_{50} values ranging from 2.84 to 8.55 μg/mL and 0.007–0.030 mM. The structures of the newly synthesized compounds were evaluated by elemental and spectral (IR, ^{1}H-NMR, ^{13}C-NMR) methods. The results demonstrated the potential and importance of developing new Mannich bases which would be effective against resistant microbial strains and may be useful leads for anticancer drug development in the future.

Keywords: benzylidene; antimicrobial; cytotoxicity; Mannich bases; ^{1}H-NMR; ^{13}C-NMR.

INTRODUCTION

The incidence of microbial infections has increased to startling levels worldwide in the last 25 years because of antimicrobial resistance. The hasty develop-

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ment of resistance to existing antibacterial and antifungal drugs poses a major threat to public health and generates a serious challenge to the scientific community. The increasing number of immuno-compromised patients as a result of cancer chemotherapy, organ transplantation and HIV infection are the major factors contributing to this increase. Consequently, there is a vital need for the development of new antimicrobial agents having potent activity against the resistant microorganism.[2–5] Hydrazones belong to the Schiff base family containing azomethine –NHN=CH– protons and the hydrazone moiety is considered one of the privileged structural fragments in modern medicinal chemistry due to its broad pharmacological vistas. Among the important pharmacophores responsible for antimicrobial activity, the hydrazone group is still considered a viable lead structure for the synthesis of more efficacious and broad-spectrum antimicrobial agents. Some widely used antibacterial drugs, such as nitrofurazone, furazolidone and ftivazide, contain the hydrazone group. Furthermore, pharmacokinetic and cellular permeability of a drug can be increased by derivatization to a bioreversible form of this drug, namely hydrazone. It is believed that the hydrazone functional group increases the lipophilicity of the parent amine and amides, which results in an enhancement of absorption through biomembranes and it is this enhanced lipophilicity of hydrazones which enables them to cross bacterial and fungal membranes.[6,7] Hydrazones are formed when hydrazines react with an aldehyde or a ketone under specific condition. Hydrazones have been reported to possess antimicrobial,[8,9] antitubercular,[10,11] antileprotic,[12] anticonvulsant,[13] analgesic,[14] anti-inflammatory,[15,16] antiplatelet,[17] anticancer[18,19] and antiviral properties.[20]

Inspired by the above facts and in continuation of an ongoing research program in the field of the synthesis and antimicrobial activity of medicinally important compounds,[21–24] the synthesis and antimicrobial activity of some novel derivatives of isoniazid are reported herein.

EXPERIMENTAL

Melting points of the synthesized compounds were determined in open-glass capillaries on a Stuart SMP10 melting point apparatus and are uncorrected. The purity of the compounds was checked by thin layer chromatography (TLC). Silica gel plates 0.25 mm, 60 GF_{254}, precoated sheets, obtained from Merck, Darmstadt (Germany), were used for the TLC and the spots were visualized by iodine vapor or ultraviolet light as visualizing agents. The infrared (IR) spectra were obtained on a Perkin-Elmer 1600 FTIR spectrometer in KBr pellets. The nuclear magnetic resonance (NMR) spectra were recorded in deuterated dimethyl sulfoxide (DMSO-d_6) solutions using tetramethylsilane as the internal reference. A Varian-Mercury 300 MHz spectrometer was used for recording the ^{1}H-NMR spectra while the ^{13}C-NMR spectra were acquired on a Bruker Avance II 400 spectrometer. Elemental analyses were performed on an ECS 4010 elemental combustion system. The necessary chemicals were purchased from Loba Chemie, Fluka and Sigma-Aldrich.

Synthesis of 2-propoxybenzylideneisonicotinohydrazide.

A mixture of 2-propoxybenzaldehyde (1.64 g, 0.010 mol) and isoniazid (1.37 g, 0.010 mol) in 15 ml of super dry ethanol was refluxed for 5 h. Completion of the reaction was confirmed by thin layer chromatography (TLC). The reaction mixture was then poured into ice-cold water and the obtained precipitate was filtered and dried in oven at a low temperature. The product was recrystallized from absolute ethanol.

*Synthesis of substituted Mannich bases (**2b**–**k**)*

2-Propoxybenzylideneisonicotinohydrazide (**1**) (679 mg, 0.0024 mol), formaldehyde (0.10 ml, 0.0036 mol) and the required substituted secondary amine (0.0024 mol) were placed in 100 ml round-bottom flask to which 50 ml of super dry ethanol was added, the pH was adjusted to 4 with hydrochloric acid and the mixture was refluxed for 28–33 h. Completion of the reaction was confirmed by TLC. The reaction mixture was allowed to cool to room temperature and then diethyl ether was added. The reaction mixture was kept for 3–5 h in a refrigerator. The resulting solid was filtered off and washed with *n*-hexane. The products were recrystallized from absolute ethanol.

Antimicrobial evaluation

The synthesized compounds were evaluated for their *in vitro* antimicrobial activity against the Gram-positive bacteria *Staphylococcus aureus* (MTCC 96), *Bacillus subtilis* (MTCC 121), Gram-negative *Escherichia coli* (MTCC 40), *Pseudomonas aeruginosa* (MTCC 2453) and the fungal strains *Candida albicans* (MTCC 227) and *Aspergillus niger* (MTCC 8189). The antimicrobial activity was assessed by the serial two-fold dilution technique. Amoxicillin was used as the standard drug for the antibacterial activity and nystatin was used as the standard drug for the antifungal activity. All the compounds were dissolved in dimethyl sulfoxide (DMSO) to give a concentration of 100 μg mL^{-1}. Twofold dilutions of the test and standard compounds were prepared in double strength nutrient broth for bacteria (Indian Pharmacopoeia: peptone 10 g, meat extracts 10 g, sodium chloride 0.5 g, distilled water 1000 mL, and pH 7.2±0.2) or Sabouraud dextrose broth for fungi (Indian Pharmacopoeia)[25]. The stock solutions were serially diluted to give concentrations of 50–0.78 μg mL^{-1} in the respective nutrient broth. The inoculum size was approximately 10^6 colony forming units (CFU) mL^{-1}. The tubes were incubated at 37±1 °C for 24 h (bacteria) and 25 °C for 7 d (*A. niger*) and 37±1 °C for 48 h (*C. albicans*). After incubation, the inoculated culture tubes were macroscopically examined for turbidity. The culture tube showing turbidity (lower concentration) and the culture tube showing no turbidity (higher concentration) gave the minimum inhibitory concentration (*MIC*) for the compound.

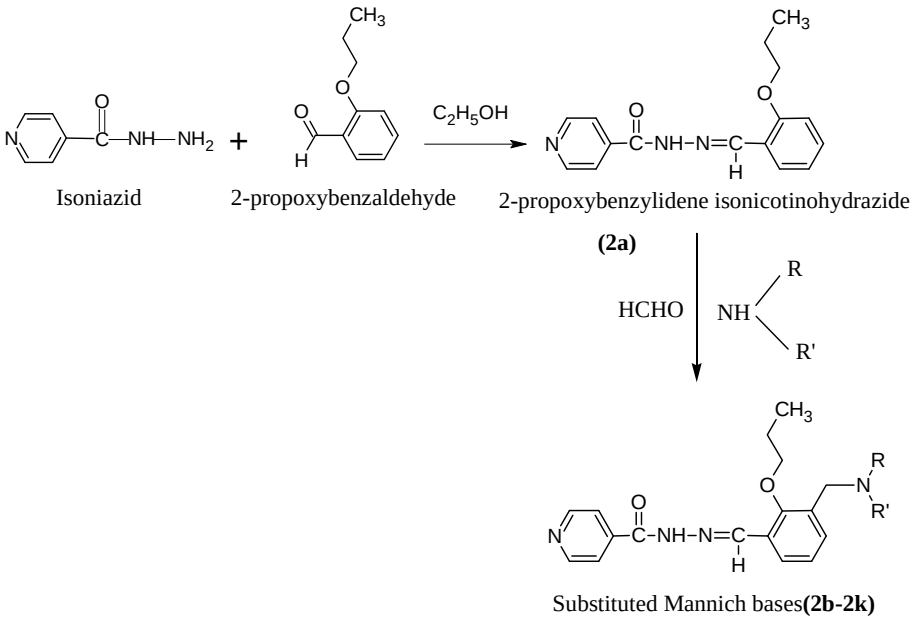
Cytotoxicity studies

The A549 (human lung adenocarcinoma) cell line was obtained from the National Centre for Cell Science, Pune, India. The cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mmol L^{-1} L-glutamine, 10 % fetal bovine serum (FBS), penicillin (50 IU mL^{-1}) and streptomycin (50 mg mL^{-1}) at a temperature of 37 °C in a humidified incubator with a 5 % CO_2 atmosphere. Gemcitabine, a well known anticancer drug, was used as the positive control for comparison.[26] The viability of the cells was assessed by the MTT 2-(5-dimethylthiazol-2-yl)-3,5-diphenyl-2*H*-tetrazolium bromide) assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product.[27] Cells (1×10^4) were placed in a 96-well plate. After 24 h, they were treated with different concentrations (0–25 μg/mL) of the test compounds diluted appropriately with culture media for 48 h. Cells grown in media containing an equivalent amount of DMSO served

as the positive control and cells in medium without any supplementation were used as the negative control. After the treatment, the media containing the compounds were carefully removed. MTT in phosphate-buffered saline (PBS, 100 μL, 0.40 mg mL^{-1}) was added to each well and incubated in the dark for 4 h. Then DMSO (100 μL) was added to each well and kept in an incubator for 4 h for dissolution of the formed formazan crystals. The amount of formazan was determined by measuring the absorbance at 540 nm using an ELISA plate reader. The data are presented as percent post treatment recovery (% of live cells), whereas the absorbance from non-treated control cells was defined as 100 % live cells.

RESULT AND DISCUSSION

The synthesis of target compounds were carried as outlined in the Scheme 1. An equimolar quantity of 2-propoxybenzaldehyde and isoniazid in 15 ml of absolute ethanol was refluxed for 7 h to form acid hydrazone. The completion of reaction was confirmed by thin layer chromatography (TLC). Then 2-propoxybenzylideneisonicotinohydrazide along with formaldehyde and secondary amines was refluxed for 34–42 h in presence of 50 ml of super dry ethanol and the pH was adjusted to 4 with hydrochloric acid. The employed secondary amines are specified in Table I.

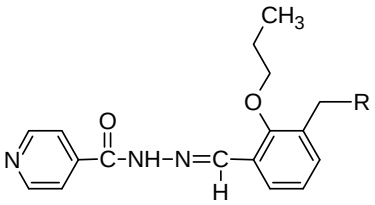


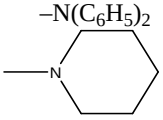
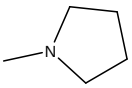
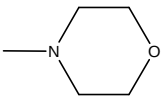
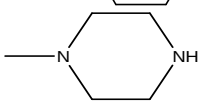
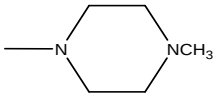
Scheme 1. General synthetic pathway for the formation of the title compounds.

The purity of the compounds was checked by TLC and they were characterized by elemental analysis and IR, ^{1}H- and ^{13}C-NMR spectroscopy. The results of the elemental analyses and the spectral data are given in the Supplementary data to this paper.

In general, the IR spectra of all compounds **2a**–**k** showed absorption band in the 3279–3255, 2978–2946, 2862–2835, 1677–1663, 1659–1641, 1581–1552, 1174–1125 and 1079–1038 cm^{-1} regions, conforming the presence of NH, CH, CH_2, C=N, C=O, C=C and C–N, respectively. The structures of the prepared derivatives were confirmed by their ^{1}H-NMR spectrum based on the chemical shifts, multiplicities, and coupling constants. The spectra of most compounds showed the characteristic NH proton at δ 11.98–11.79 ppm, the proton of –N=C–H at δ 8.79–8.21 ppm, the 4 protons of pyridine at around δ 8.86–7.46 ppm, the characteristic protons of benzylidene at δ 7.77–6.78 ppm, the 2 protons of Ar–O––C**H**$_2$ at δ 3.94–3.62 ppm and the 2 protons of Ar–C**H**$_2$–N at δ 3.69–3.49 ppm, The ^{13}C-NMR spectra of most compounds had characteristic signals of C=O at around δ 163.91–163.18 ppm, of pyridine at δ 149.88–122.15 ppm, of the –N=C–H group at δ 143.48–143.19 ppm, of benzylidene at δ 157.58–115.75 ppm and of Ar–O–**C**H$_2$ at δ 72.84–72.19 ppm and Ar–**C**H$_2$–N at δ 55.89–45.59 ppm.

TABLE I. Structure and physical data of the synthesized Mannich bases



Compound	R	Molecular formulae	Yield, %	M.p., °C
2b	–N(CH$_3$)$_2$	$C_{19}H_{24}N_2O_2$	48	227–230
2c	–N(C$_2$H$_5$)$_2$	$C_{21}H_{28}N_4O_2$	45	235–238
2d	–N(C$_3$H$_7$)$_2$	$C_{23}H_{32}N_4O_2$	52	212–215
2e	–N(C$_4$H$_9$)$_2$	$C_{25}H_{36}N_4O_2$	43	218–221
2f	–N(C$_6$H$_5$)$_2$	$C_{29}H_{28}N_4O_2$	39	182–185
2g		$C_{22}H_{28}N_4O_2$	48	188–191
2h		$C_{21}H_{26}N_4O_2$	45	193–196
2i		$C_{21}H_{26}N_4O_3$	40	223–226
2j		$C_{21}H_{27}N_5O_2$	43	205–208
2k		$C_{22}H_{29}N_5O_2$	46	117–120

The compounds were evaluated for their antimicrobial properties in comparison the control antibacterial agent amoxicillin and antifungal agent nystatin.

The results of antibacterial screening, Table II, revealed that all the tested compounds showed moderate to good bacterial inhibition. Compounds **2c** and **2k** displayed excellent activity against the microbial strains. Compounds **2d, 2h, 2i**

TABLE II. Antimicrobial screening results (*MIC* / μg mL^{-1}) of the tested compounds

Compound	Gram-positive bacteria		Gram-negative bacteria		Fungal strain	
	B. subtilis	*S. aureus*	*P. aeruginosa*	*E. coli*	*C. albicans*	*A. nigar*
2a	25	12.5	12.5	6.25	25	12.5
2b	6.25	12.5	3.12	6.25	25	>100
2c	0.78	1.56	1.56	3.12	1.56	3.12
2d	3.12	12.5	6.25	25	12.5	12.5
2e	25	>100	12.5	6.25	3.12	12.5
2f	12.5	6.25	12.5	12.5	6.25	>100
2g	12.5	25	25	12.5	12.5	6.25
2h	6.25	25	12.5	6.25	12.5	25
2i	6.25	6.25	12.5	12.5	12.5	3.12
2j	6.25	12.5	6.25	12.5	12.5	6.25
2k	3.12	6.25	1.56	1.56	12.5	25
Amoxicillin	0.15	0.15	0.25	0.15	–	–
Nystatin	–	–	–	–	0.25	0.78

and **2j** showed moderate antibacterial activity. Of all the synthesized derivatives, compound **2a** was found to be the least active compound against most of bacterial strains. Concerning the antifungal activity of the tested compounds, only two fungal strains were selected, *C. albicans* and *A. niger*. The data of the antifungal screening, Table II, revealed that all the tested compounds showed moderate to good fungal inhibition as compared to standard drug nystatin. Among the derivatives, compound **2c** exhibited the highest antifungal activity against both fungal strains, while compounds **2e, 2g** and **2j** showed moderate antifungal activity. Of all the synthesized derivatives, compound **2b** was found to be the least active compound against both fungal strains.

In addition, all the tested compounds demonstrated remarkable cytotoxicity against A549 lung cancer cell line, Table III. In particular, compounds **2c** and **2k** displayed significant inhibitory activity superior to that of the reference compound gemcitabine.

TABLE III. The cytotoxicity data against the A549 lung cancer cell line of the synthesized compounds

Compound	Cell death at various doses (in μg/mL), %					IC_{50}[a] μg mL^{-1}	IC_{50} mM
	1	2	5	10	25		
2a	3.13	17.55	30.85	47.62	60.81	8.55	0.030
2b	4.19	18.13	31.18	48	61.28	8.12	0.023
2c	10.54	30.73	41.18	73.48	77.29	2.84	0.007
2d	9.12	23.26	36.55	69.84	71.39	4.18	0.010
2e	5.83	19.63	32.15	60.45	65.64	6.58	0.015
2f	4.15	18.25	31.52	51.13	62.81	7.73	0.016
2g	4.85	18.33	31.87	55.18	64.35	7.15	0.018
2h	9.15	21.35	34.16	66.65	70.19	5.18	0.014
2i	8.87	20.17	32.55	63.72	69.39	5.92	0.015
2j	8.22	19.77	32.12	61.15	66.29	6.29	0.016
2k	9.53	25.23	39.19	71.19	74.11	3.36	0.008
Gemcitabine	6.53	15.18	25.93	54.58	71.18	6.19	0.023

[a]The half maximal (50%) inhibitory concentration (IC) of a substance

CONCLUSIONS

In conclusion, the present paper describes the preparation and biological evaluation of a series of novel isoniazid derivatives. The synthesized compounds were characterized by suitable analytical techniques, *i.e.*, IR, ^{1}H-NMR, ^{13}C-NMR and elemental analysis and the data obtained was in full agreement of the proposed structures. Among the synthesized derivatives, compound **2c** and **2k**, having a diethylamino and methylpiperazine moieties, were the most active compounds with significant biological activity. Based on preliminary antimicrobial and cytotoxicity results, it is supposed that both these compounds display antimicrobial and cytotoxicity activities through a non-specific mechanism of action or that they exert antimicrobial action due to their cytotoxicity activity. Taken together, the compounds reported in this paper could serve as potential leads for future studies and that further investigations are warranted to determine the mechanism of action of these molecules.

SUPPLEMENTARY MATERIAL

Analytical and spectral data of synthesized compounds are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

ИЗВОД

СИНТЕЗА И ИСПИТИВАЊЕ АНТИМИКРОБНЕ АКТИВНОСТИ НЕКИХ НОВИХ ДЕРИВАТА 2-ПРОПОКСИБЕНЗИЛИДЕН-ИЗОНИКОТИНОХИДРАЗИДА

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Синтетисана је серија нових Манихових база, деривата изонијазида. Прво је реакцијом 2-пропоксибензалдехида са изонијазидом добијен одговарајући хидразон (**2a**). Производ **2a** је у Маниховој реакцији аминометиловања формалдехидом и секундарним аминима дао производе (**2b**–**k**). Инхибиторна *in vitro* активност добијених једињења испитана је према панелу микроорганизама и A549 ћелијској линији аденокарцинома плућа. Деривати **2c** и **2k** имају умерену активност према микроорганизмима, и показују значајну цитотоксичност која зависи од примењене концентрације једињења. IC_{50} вредности налазе се у опсегу 2,84–8,55 μg/mL и 0,007–0,030 mM. Структуре синтетисаних једињења одређене су на основу резултата елементалне анализе и спектралних података (IC, ^{1}H-NMR и ^{13}C-NMR). Добијени резултати показују на значај развоја Манихових база, које могу бити активне према резистентним сојевима микроорганизама, и могу бити развијани као успешни модели за развој антитуморских једињења.

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REFERENCES

1. M. Koca, S. Servi, C. Kirilimis, M. Ahmedzade, C. Kazaz, B. Ozbek, G. Otuk, *Eur. J. Med. Chem.* **40** (2005) 1351
2. C. Bonde, N. J. Gaikwad, *Bioorg. Med. Chem.* **12** (2004) 2151
3. D. Yu, G. Huiyuan, *Bioorg. Med. Chem. Lett.* **12** (2002) 857
4. S. D. Joshi, H. M. Vagdevi, V. P. Vaidya, G. S. Gadaginamath, *Eur. J. Med. Chem.* **43** (2008) 1989
5. S. Rollas, S. G. Kucukguzel, *Molecules*. **12** (2007) 1910
6. S. T. Murphy, H. L. Case, E. Ellsworth, S. Hagen, M. Husband, T. Jonnides, C. Limberakis, R. Marotti, A. M. Ottolini, M. Rauckhorst, J. Starr, M. Stier, C. Taylor, T. Zhu, A. Blasser, W. A. Denny, G. L. Lu, J. B. Smailic, F. Rivault, *Bioorg. Med. Chem. Lett.* **17** (2007) 2150
7. T. Scior, S. J. Garces-Eisele, *Curr. Med. Chem.* **13** (2006) 2205
8. S. Rollas, N. Gulerman, H. Edinz, *Farmaco* **57** (2002) 171
9. A. Imramovsky, S. Polanac, J. Vinsova, M. Kocevar, J. Jampitek, Z. Reckova, J. A. Kaustova, *Bioorg. Med. Chem.* **15** (2007) 4229
10. M. J. Hearn, M. H. Cynamon, M. F. Chen, R. Coppins, J. Davis, H. O. J. Kang, A. Noble, B. T. Sekine, M. S. Terrot, D. Trombino, M. Thai, E. R. Webster, R. Wilson, *Eur. J. Med. Chem*. **44** (2009) 4169
11. S. J. Gilani, S. A. Khan, N Siddiqui, *Bioorg. Med. Chem. Lett.* ***20*** **(**2010) 4762

12. F. R. Pavan, P. I. D. S. Maia, S. R. Leite, V. M. Deflon, A. A. Batista, D. N. Sato, S. G. Franzblau, C. Q. Leite, *Eur. J. Med. Chem*. **45** (2010) 1898
13. R. Sinha, U. V. S. Sara, R. L. Khosa, J. Stables, J. Jain, *Med. Chem. Res.***20** (2011) 1499
14. U. Salgin-Goksen, N. Gokhan-Kelekci, O. Goktas, Y. Koysal, E. Kılıc, S. Isik, G. Aktay, M. Ozalp, *Bioorg. Med. Chem.* **15** (2007) 5738
15. M. Malhotra, S. Sharma, A. Deep, *Med. Chem. Res.* **21** (2012) 1237
16. G. A. Silva, L. M. M. Costa, F. C. F. Brito, A. L. P. Miranda, E. J. Barreiro, C. A. M. Fraga, *Bioorg. Med. Chem*. **12** (2004) 3149
17. L. Savini, L. Chiasserini, V. Travagli, C. Pellerano, E. Novellino, S. Consentino, M. B. Pisano, *Eur. J. Med. Chem*. **39** (2004) 113
18. A. Bijev, *Lett. Drug Des. Discovery* **3** (2006) 506
19. C. Loncle, J. M. Brunel, N. Vidal, M. D. Herbomez, Y. Letourneux, *Eur. J. Med. Chem.* **39** (2004) 1067
20. M. T. Abdel-Aal, W. A. El-Sayed, E. H. El-Ashry, *Arch. Pharm. Chem. Life Sci.* **339** (2006) 656
21. A. Deep, S. Jain, P. C. Sharma, P. Verma, M. Kumar, C. P. Dora, *Acta Pol. Pharm.* **67** (2010) 255
22. A. Madhukar, N. Kannappan, A. Deep, P. Kumar, M. Kumar, P. Verma, *Int. J. ChemTech Res.* **1** (2009) 1376
23. M. Kumar, S. Jain, A. Deep, *Lat. Am. J. Pharm.* **30** (2010) 388
24. A. Deep, S. Jain, P. C. Sharma, S. K. Mittal, P. Phogat, M. Malhotra, *Arab. J. Chem.* (2011) doi: 10.1016/j.arabjc.2010.10.032
25. *Pharmacopoeia of India*, Vol. II, Ministry of Health Department, Goverment of India, New Delhi, 1996, p. A-88
26. R. H. Kenneth, P. J. Wedlund, R. M. Noone, G. R. Wilkinson, F. A. Greco, S. N. Wolff, *Cancer Res*. **44** (1984) 379
27. T. Mossman, *J. Immunol. Methods* **65** (1983) 55.