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SYNTHESIS, CHARACTERIZATION AND EVALUATION OF ANTICANCER ACTIVITY OF SOME 7-(SUBSTITUTED BENZYLIDENE)-3-ARYL-2, 3, 4, 5, 6, 7-HEXAHYDRO INDAZOL-1-YL (PYRIDIN-4-YL) METHANONES

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ABSTRACT

Objective: Colorectal cancer (CRC) is the third most frequently occurring cancer in both male and females, but it ranks second in developed countries. The incidence of colorectal cancer is still increasing in large parts of the world due to the development of resistance to the available drugs. Thus, there is a need to develop novel colorectal anti cancer agents. **Materials and Methods:** The present work includes the synthesis of a series of new 7-(Substituted benzylidene)-3-aryl-2, 3, 4, 5, 6, 7-hexahydroindazol-1-yl(pyridine-4-yl)methanones from substituted chalcones. These chalcones were prepared by Claisen-Schmidt reaction by the condensation of cyclohexanone and various substituted aromatic aldehydes. The synthesized compounds were confirmed by IR, ^{1}H NMR, MASS spectra and CHNO Elemental analysis. The synthesized compounds were predicted for biological activities by *Insilico* method based on those predictions the compounds screened for colorectal anti cancer activity (MTT assay) by using cell line HT-29 human colorectal adenocarcinoma using cisplatin as standard drug. **Results:** The compounds 2a, 2c, 2f, 3a, 3c and 3f exhibited maximum activity at a concentration <10 µg, 2b, 2e, 3b and 3e exhibited moderate activity at a concentration <20 µg. Remaining compounds 2d and 3d exhibited no activity at concentration >30 µg. **Conclusion:** The synthesized compounds having the electron releasing groups such as hydroxyl and dimethyl amino group in phenyl ring exhibited maximum activity. Hence it clearly indicates the importance of electron releasing groups on aromatic rings for anticancer activity.

Keywords: Chalcones, Indazoles, Colorectal cancer, HT-29 cell line.

INTRODUCTION

Colorectal cancer (CRC) is the third most frequently occurring cancer in both male and females, but it ranks second in developed countries. More than 80% of CRC cases arise from adenomatous polyps. Less than 1% of adenomatous polyps smaller than 1cm become cancer.

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Despite the development of several types of synthetic colorectal anti cancer agents, the incidence of colorectal cancer is still increasing in large parts of the world due to the development of resistance to the available drugs. Thus, there is an urgent need for novel colorectal anti cancer agents with modes of action and chemical structure different from the currently used compounds, it is planned to synthesize new indazole moieties.

Indazoles constitute an important class of heterocycles that display interesting biological properties such as anti-depressant [1], anti-inflammatory [2, 3], analgesic, anti-pyretic [4], dopamine antagonistic [5], anti-tumor [6], anti-emetic [7] and anti-HIV [8] activities. The indazole ring system is also present in many

other compounds such as herbicides, dyes or sweeteners like guanidine-1H indazole. Among the heterocyclic compounds available for the preparation of potentially valuable new building-blocks in medicinal chemistry, the indazole nucleus is probably one of the least studied. In contrast to the abundance of publications based on its bioisosteres e.g. indole [9, 10], benzimidazole [11, 12], there are a limited number of publications based on indazole chemistry, presumably a result of the fact that indazole moiety is rare in natural products [13-15]. Even so, a large number of synthetically prepared indazoles have displayed biological and pharmacological properties. [16]

MATERIALS AND METHODS

Melting points of the synthesized compounds were determined in an open capillary tube using digital melting point apparatus and are uncorrected. The purity of the synthesized compounds were established by thin layer chromatography by using pre-coated silica gel strips, solvent system used is chloroform and acetone (2:1) and spots were detected by visualizing agent iodine vapor. Infrared spectra (υ cm^{-1}) were recorded on a SHIMADZU FT-IR 4000 using KBr disks. Mass spectra were obtained on JEOL GC mate II GC- Mass spectrometer at 70ev using direct insertion probe method. CHNO elemental analysis carried by Perkin Elmer Series II 2400 CHNS/O Elemental analyzer. ^{1}H NMR spectra were taken on BRUKER AV400-400MHz High resolution multinuclear FT-NMR spectrometer by using TMS as internal standard and the solvents used was DMSO and $CDCl_3$.

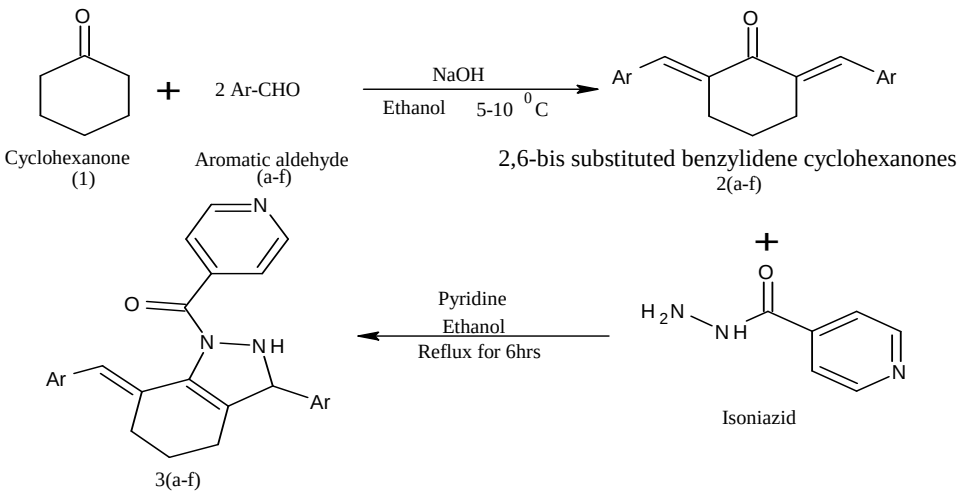
EXPERIMENTAL WORK:

The synthesis of various 7-(substituted benzylidene)-3-(substituted phenyl)-2,3,4,5,6,7–hexahydroindazol-1-yl)(pyridine-4-yl) methanones (indazoles) start with the reaction of cyclohexanone (1) and substituted aromatic aldehydes (a-f) by Claisen-Schmidt reaction to yield 2,6-bis substituted benzylidene cyclohexanones - chalcones (2a-f). These synthesized 2,6-bis substituted benzylidene cyclohexanones refluxed with isoniazid in presence of pyridine by nucleophilic attack to get 7-(substituted benzylidene)-3-(substitutedphenyl)-2, 3, 4, 5, 6, 7 –hexahydroindazol-1-yl) (pyridine-4-yl) methanones – indazoles (3a-f). Completion of the reactions was determined by thin layer chromatography by using chloroform and acetone as mobile system.

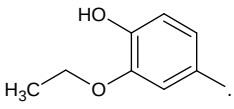
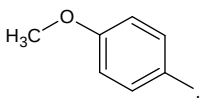
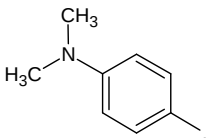
General procedure for the synthesis of 2,6-bis substituted benzylidene cyclohexanones (2a-f):

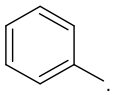
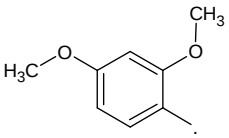
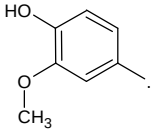
A mixture of cyclohexanone (1) (1mL, 0.01mol) and substituted aromatic aldehydes (0.02mol) in ethanol (50mL) was cooled at 5-10^{0}C, to this add aqueous sodium hydroxide solution (70%, 5mL) drop wise with constant stirring. Then the reaction mixture was further stirred for 2hrs and left over night and neutralized with concentrated hydrochloric acid. The solid then separated was filtered and recrystallized from ethanol.

General scheme of the work:



Various aromatic aldehydes (Ar) in 2a-f and 3a-f:

a  b  c 

d  e  f 

2, 6-bis (3-ethoxy-4-hydroxybenzylidene) cyclohexanone (2a):

Yield-89%, m.p. 121-125^{0}C; IR (υ cm-1) spectrum showed characteristic bands at 1714.93(C=O), 1867.32 (C-H aromatic out of plane summation bands), 1635.83(C=C), 1267.39 [C-O (aryl)], 1041.69(C-O (alkyl)], 3591.89 (C-OH stretching), 2972.67 (C-H str), 1446.79 (C-H def). Calcd for $C_{24}H_{26}O_5$: C, 73.08; H, 6.64; O, 20.07. Found: C, 73.05; H, 6.66; O, 20.08.

2, 6-bis (4-methoxybenzylidene)cyclohexanone (2b):

Yield-93%, m.p. 191-195^{0}C; IR (υ cm-1) spectrum showed characteristic bands at 1250.02 [C-O (aryl)], 1062.91 [C-O (alkyl)], 1448.72 (C-H def), 1716.85(C=O), 1822.95(C-H aromatic out of plane summation bands),1657.05(C=C), 2833.78 [C-H (OCH_3)]; ^{1}H NMR (400MHz, DMSO) δ 7.49 (2H, s, CH_2), δ 6.7-8.3 (8H, m, aromatic), δ 3.295-3.79 (6H, m, CH_3); Mass m/z 334.76; Anal. Calcd for $C_{22}H_{22}O_3$: C, 79.02; H, 6.63; O, 14.35. Found: C, 79.04; H, 6.64; O, 14.37.

2,6-bis(4-(dimethylamino)benzylidene)cyclohexanone (2c):

Yield-91%, m.p. 203-205^{0}C; IR (υ cm-1) spectrum showed characteristic bands at 1714.93(C=O), 1867.32 (C-H aromatic out of plane summation bands), 1635.83(C=C), 1267.39 [C-O (aryl)], 1041.69(C-O (alkyl)], 3591.89 (C-OH stretching), 2972.67 (C-H str), 1446.79 (C-H def). Calcd for $C_{24}H_{28}N_2O$: C, 79.96; H, 7.83; O, 4.44; N, 7.77. Found: C, 79.93; H, 7.85; O, 4.46; N, 7.76.

2,6-dibenzylidenecyclohexanone (2d):

Yield-92%, m.p. 130-136^{0}C; IR (υ cm-1) spectrum showed characteristic bands at 1705 (C=O), 1950 (C-H aromatic out of plane summation bonds), 1660 (C=C), 1485 [C-H def ($-CH_2$)]; ^{1}H NMR (400MHz, DMSO) δ 7.830 (2H, s, C_2H_4), δ 7.350-7.530 (10H, m, aromatic protons), δ 2.949-2.978 (4H, m, CH_2); Mass m/z 274.3563; Calcd for $C_{20}H_{18}O$: C, 87.56; H, 6.61; O, 5.83. Found: C, 87.59; H, 6.63; O, 5.85.

2, 6-bis (3, 4-dimethoxybenzylidene)cyclohexanone (2e):

Yield-88%, m.p. 161-165^{0}C; IR (υ cm-1) spectrum showed characteristic bands at 1714 (C=O), 1460 (C-H def), 1660 (C=C), 2833 [C-H str ($-OCH_3$)], 2930 ($-CH_2$ str); ^{1}H NMR (400MHz, DMSO) δ 7.753 (2H, s, C_2H_4), δ 6.900-7.261 (6H, m, aromatic protons), δ 3.867-3.924 (12H, m, CH_3) δ 2.932-2.967 (4H, m, CH_2); Mass m/z 394.100; Calcd for $C_{24}H_{26}O_5$: C, 73.08; H, 6.64; O, 20.28. Found: C, 71.32; H, 6.01; O, 19.87.

2,6-bis(3,4-dimethoxybenzylidene)cyclohexanone (2f):

Yield-74%, m.p. 96-100^{0}C; IR (υ cm-1) spectrum showed characteristic bands at 1714 (C=O), 1653 (C=C), 1458 [C-H def ($-CH_2$)], 2920 [C-H str ($-CH_2$)], 1683 (aromatic summation bonds), 3601(-OH str); Calcd for $C_{22}H_{22}O_5$: C, 72.12; H, 6.05; O, 21.83. Found: C, 72.16; H, 6.02; O, 21.80.

(7-(3-ethoxy-4-hydroxybenzylidene)-3-(3-ethoxy-4-hydroxyphenyl)- 2,3,4,5,6,7-hexahydroindaz ol-1-yl)(pyridin-4-yl)methanone (3a):

Yield-88%, m.p. 252-255^{0}C; IR (υ cm-1) spectrum showed characteristic bands at 1633.91(C=O) , 1331.04 [C-N(3^{0}amide)], 1633.91 (N-H), 1616.54 [C-H str($-CH_2$)], 1456.43 [C-H def($-CH_2$)], 1842.24(C-H aromatic

out of plane summation bands), 1230.73 [C-O (aryl)], 3595.75 (OH-), 2963.02 [C-H str (-CH_3)], 1437.14 [C-H def(-CH_3)]. Calcd for $C_{30}H_{31}N_3O_5$: C, 70.16; H, 6.08; N, 8.18; O, 15.58. Found: C, 70.18; H, 6.10; N, 8.16; O, 15.56.

(7-(4-methoxybenzylidene)-3-(4-methoxyphenyl)-2, 3, 4, 5, 6, 7-hexahydroindazol-1-yl)(pyridin-4-yl) methanone (3b):

Yield-85%, m.p. 141-145^{0}C; IR (υ cm-1) spectrum showed characteristic bands at 1657.05(C=O), 1313.68 [C-N(3^{0}amide)], 1570.25 [N-H(2^{0}amine)],1657.05(C=C), 2932.16 [C-H(-CH2)], 1458.36 [C-H (CH2)],1846.10(C-H aromatic out of plane summation bands), 1255.81 [C-O (aryl)], 1059.05 [C-O(alkyl)], 2820.27 [C-H(-OCH3)]; ^{1}H NMR (400MHz, DMSO) δ 2.490 (1H, s, NH), δ 7.013-8.406 (8H, m, aromatic protons), δ 7.96-9.06 (4H, m, C_5H_5N), δ 2.486-3.806 (6H, s, CH_3), δ 7.013 (2H, s, CH_2); Mass m/z 453.28; Calcd for $C_{28}H_{27}N_3O_3$: C, 74.15; H, 6.00; O, 10.58; N, 9.27. Found: C, 74.13; H, 6.02; O, 10.55; N, 9.29.

(7-(4-(dimethylamino) benzylidene)-3-(4-(dimethylamino)phenyl)-2,3,4,5,6,7-hexahydroindazol-1-yl)(pyridin-4-yl) methanone (3c):

Yield-95%, m.p. 240-244^{0}C; IR (υ cm-1) spectrum showed characteristic bands at 1662.84(C=O), 1359.98 [C-N (3^{0}amide)], 1579.89[N-H(2^{0}amine)], 2924.44[C-H(-CH_2)], 1444.86[C-H def (-CH_2)], 1886.61(C-H aromatic out of plane summation bands), 2955.31 [C-H str(-CH_3)], 1431.36 [C-H def (-CH_3)]; Calcd for $C_{30}H_{33}N_5O$: C, 75.13; H, 6.94; O, 3.34; N, 14.60. Found: C, 75.16; H, 6.92; O, 3.35; N, 14.58.

7-(benzylidene-3-phenyl-2,3,4,5,6,7-hexahydroindazol-1-yl)(pyridin-4-yl) methanone (3d):

Yield-83%, m.p. 193-199^{0}C; IR (υ cm-1) spectrum showed characteristic bands at 1660.91(C=O), 1367.70 [C-N (3^{0}amide)], 1660.91(C=C), 2937.94 [C-H (-CH_2)], 1446.79 [C-H def (-CH_2)], 1747.72 (C-H aromatic out of plane summation bands), 1805.59 [N-H 2^{0}amine]; ^{1}H NMR (400MHz, DMSO) δ 1.683-1.859 (3H, s, CH_2), δ 7.39-7.550 (10H, m, aromatic), δ 2.506 (1H, s, NH), δ 7.755-7.844 (4H, m, C_5H_5N), δ 7.411 (1H, s, CH_2); Mass m/z 393.7196; Calcd for $C_{26}H_{23}N_3O$: C, 79.36; H, 5.89; O, 4.07; N, 10.68. Found: C, 79.38; H, 5.88; O, 4.09; N, 10.66.

(7-(3,4-dimethoxybenzylidene)-3-(3,4-dimethoxyphenyl)-2,3,4,5,6,7-hexahydroindazol-1-yl) (pyririn-4-yl)methanone (3e):

Yield-92%, m.p. 201-206^{0}C; IR (υ cm-1) spectrum showed characteristic bands at 1579.89 [N-H (2^{0}amine)], 1657.05 (C=O), 1805.59(C-H aromatic out of plane summation bands), 1657.05 (C=C), 1466.08 [C-H def (-CH_2)], 1331.04 [C-N (3^{0}amide)], 2837.63(C-H (-OCH_3)], 1280.89(C-O); ^{1}H NMR (400MHz, DMSO) δ 1.714 (3H, s, CH_2), δ 3.847-3.973 (12H, s, CH_3), δ 6.846-7.118 (6H, m, aromatic), δ 7.966-9.685 (4H, m, C_5H_5N), δ 6.866 (1H, s, CH_2); Mass m/z 513.1031; Calcd for $C_{30}H_{31}N_3O_5$: C, 70.16; H, 6.08; O, 15.58; N, 8.18. Found: C, 70.18; H, 6.07; O, 15.56; N, 8.16.

(7-(4-hydroxy-3-methoxybenzylidene)-3-(4-hydroxy-3-methoxyphenyl)-2,3,4,5,6,7-hexahydro indazol-1-yl)(pyridin-4-yl)methanone (3f):

Yield-91%, m.p. 215-218^{0}C; IR (υ cm-1) spectrum showed characteristic bands at 1585.68 [N-H (2^{0}amine)], 1643.55(C=O), 1844.17 (C-H aromatic out of plane summation bands), 1643.55 (C=C), 2936.10 [C-H (-CH_2)], 1458.36 [C-H def (-CH_2)], 2829.92 [C-H (-OCH_3)], 1271.24 [C-O (aryl)], 1028.18 [C-O (alkyl)], 1329.12 [C-N (3^{0}amide)]; ^{1}H NMR (400MHz, DMSO) δ 1.362-1.379 (3H, s, CH_2), δ 3.733 (6H, s, CH_3), δ 2.026 (1H, s, NH), δ 6.387-6.781 (6H, m, aromatic), δ 7.873-9.031 (4H, s, C_5H_5N); Mass m/z 485.8165; Calcd for $C_{28}H_{27}N_3O_5$: C, 69.26; H, 5.61; O, 16.48; N, 8.65. Found: C, 69.28; H, 5.64; O, 16.45; N, 8.63.

Prediction of biological activity:

In silico prediction of biological activity in relation to the chemical structure of a compound is now a commonly used technique in drug discovery and development. It is possible with computer program PASS - Prediction of Activity Spectra for Substances, to predict the biological activity spectrum for a compound on the basis of its structural formula. It helps in finding most probable new leads with

required activity spectra among the compounds from in-house and commercial data bases. So, all the title compounds (2a-f and 3a-f) SMILES notations were entered in PASS software, among those possible biological activities the compounds showed more probability to be active for anti-neoplastic (colorectal cancer), anti-inflammatory and Alzheimer's disease treatment.[17] Data presented in table 1.

Table 1: Predicted biological activities by PASS

Code	Anti-neoplastic (colorectal cancer)		Anti-inflammatory		Alzheimer's disease treatment	
	Pa	Pi	Pa	Pi	Pa	Pi
2a	0.543	0.096	0.494	0.059	0.372	0.015
2b	0.341	0.053	0.564	0.033	0.597	0.033
2c	0.523	0.011	0.408	0.025	0.424	0.116
2d	0.496	0.026	0.632	0.008	0.473	0.015
2e	0.536	0.079	0.542	0.039	0.554	0.034
2f	0.566	0.080	0.524	0.045	0.584	0.023
3a	0.505	0.018	0.496	0.058	---	---
3b	0.331	0.057	0.521	0.046	---	---
3c	0.451	0.080	---	---	0.402	0.135
3d	0.353	0.014	0.518	0.048	0.516	0.054
3e	0.474	0.041	0.523	0.046	0.539	0.042
3f	0.490	0.019	0.506	0.053	---	---

Pa = probability "to be active"; Pi = probability "to be inactive"

COLORECTAL ANTI-CANCER ACTIVITY:

All the synthesized compounds were evaluated for their colorectal anti-cancer activity employing HT-29 human colorectal cancer cell line. The cell lines were maintained in 96 wells micro titer plate containing MEM media supplemented with 10% heat and inactivated fetal calf serum (FCS), containing 5% of mixture of Gentamycin, Penicillin (100 Units/mL) and Streptomycin (100µg/mL) in presence of 5% CO_2 at 37ºC for 3-4 days. After 3-4 days the supernatant was removed. The MEM media was replaced with Hank's balanced salt solution supplemented with Gentamycin, Penicillin and Streptomycin and incubated for overnight.

Invitro growth inhibition effect of test compound was assessed by colorimetric or spectrophotometric determination of conversion of MTT into "Formazan blue" by living cells. The supernatant was removed from the plate and added fresh Hank's balanced salt solution and it was treated with different concentration of test compound appropriately diluted with DMSO. Control group contains only DMSO. After 24 hrs incubation at 37ºC in a humidified atmosphere of 5% CO_2, the medium was replaced with MTT solution (100µL, 1mg per mL in sterile Hank's balanced salt solution) for further 4 hr incubation. The supernatant was carefully aspirated and the precipitated crystals of "Formazan blue' were solubilised by adding DMSO (200µL). The optical density (OD) was measured at wavelength of 492 nm. The result was represented by calculated mean of three readings. The concentration at which the OD of treated cells was reduced by 50% with respect to the untreated control is calculated by using the formula.

$$\text{Surviving cells (\%)} = \frac{\text{Mean OD of test compound}}{\text{Mean OD at control}} \times 100$$

This is a colorimetric assay it measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it reduces to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg. DMSO, Isopropanol) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.[18]

Table 2: Colorectal anticancer activity of synthesized compounds

S. No.	Compound code	Concentration (µg)	O.D. at 492nm	% of cell lysis	IC50
1.	2a	10	0.789	>50%	<10 µg
		20	1.232	>75%	
		30	1.865	100%	
2.	2b	10	0.889	No lysis	>20 µg
		20	1.367	No lysis	
		30	1.988	No lysis	
3.	2c	10	0.793	>50%	<10 µg
		20	1.162	>75%	
		30	1.756	100%	
4	2d	10	0.389	No lysis	>30 µg
		20	0.467	No lysis	
		30	0.488	No lysis	
5	2e	10	0.852	>50%	<20 µg
		20	1.214	>75%	
		30	1.705	100%	
6	2f	10	0.730	>50%	<10 µg
		20	1.132	>75%	
		30	1.704	100%	
7.	3a	10	0.768	>50%	<10 µg
		20	1.231	>75%	
		30	1.845	100%	
8.	3b	10	0.889	No lysis	>20 µg
		20	1.267	No lysis	
		30	1.888	No lysis	
9.	3c	10	0.776	>50%	<10 µg
		20	1.141	>75%	
		30	1.735	100%	
10.	3d	10	0.389	No lysis	>30 µg
		20	0.467	No lysis	
		30	0.488	No lysis	
11.	3e	10	0.834	>50%	<20 µg
		20	1.254	>75%	
		30	1.885	100%	
12.	3f	10	0.723	>50%	<10 µg
		20	1.115	>75%	
		30	1.716	100%	
13.	Control	-	0.507	No lysis	-

Standard drug used in colorectal anticancer activity was CISPLATIN - 10µg/mL.

RESULTS AND DISCUSSION

A facile method has been devised to synthesize the title compounds. The methods include mild conditions and yields were satisfactory. The proposed reaction led to the expected products and in all cases the products were obtained in pure form. However they were purified by recrystallization from ethanol. All the title molecules were predicted for their biological activity by using computer program PASS. The results of this prediction show colorectal anticancer activity for title compounds and were given in Table No 1; All the synthesized compounds were evaluated for their colorectal anticancer activity employing HT-29 human colorectal cancer cell line (MTT assay). Cisplatin was taken as a reference compound for comparison. The colorectal anticancer activity result was given in Table No 2. From the results, it was observed that compounds 2a, 2c, 2f, 3a, 3c and 3f exhibited maximum activity at a concentration <10µg.

The compounds 2b, 2e, 3b and 3e exhibited moderate activity at a concentration <20 µg. Rest of the compounds 2d and 3d exhibited no activity at concentration >30 µg.

CONCLUSION

All the title compounds were synthesized, characterized and screened for colorectal anti cancer activity. The results of colorectal anticancer activity revealed that some of the title compounds exhibited significant activity. It is interesting to note that compounds 2a, 2f, 3a and 3f having phenolic hydroxy group at 4th position of phenyl rings and alkoxy groups (ethoxy in 2a and 3a, methoxy in 2f and 3f) groups at 3rd position of phenyl rings possessed maximum activity. Compounds 2c and 3c having dimethylamino group at 4th position of phenyl rings also exhibited maximum activity. It clearly indicates the favorable effects of electron releasing groups on anticancer activity. The study revealed the necessity of synthesizing many more compounds having other electron releasing substituents. Such compounds may emerge as much more potent colorectal anticancer compounds.

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