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ADME Analysis of Crude Extracts of *Exacum bicolor* Roxb. and *in vitro* Screening for Antioxidant, Antimicrobial and Cytotoxicity Assays

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With the increase in medical pandemics caused by emerging and re-emerging microbial pathogens, topping multi drug resistance, there is always a dire requirement for the novel drugs to treat diseases. In view of the imperative need for lead molecules addressing ailments, the authors have extended their phytochemical studies on the crude extracts of *Exacum bicolor* Roxb, to *in silico* ADME analysis followed by *in vitro* screening for biological activities. In present study, the crude extracts of *E. bicolor* are subjected to GC-MS analysis and a total of 32 phytoconstituents were identified and considered for ADME analysis by Swiss ADME software. This study represents the first-time reports on the *in silico* screening of the plant extracts of *Exacum bicolor*. Further preliminary screening of the crude extracts displayed moderate antioxidant, cytotoxic, antibacterial and antifungal properties, which might be due to the presence of phenolic and alkaloid derivatives.

Keywords: *Exacum bicolor* Roxb., Antimicrobial, ADME, Antioxidant activity, Cytotoxicity.

INTRODUCTION

The fate of pharmaceutical molecule inside the organism can be studied by carrying out *in silico* ADME analysis [1,2]. It helps in eliminating the weak molecules and identifies the safety and efficacy of the potential drug like molecules. It also helps in reducing the cost incurred during the designing and production of pharmaceutical drugs. Hence, it is a significant part of drug regulatory approval process [3]. ADME studies also help us in assessing the safety and effective use of the drug in patients [4]. Currently, the drug discovery and development process in the pharmaceutical sector have estimated as less than 10% of molecules pass the clinical testing process [5,6]. So, ADME studies are imperative for screening of drugs for pre-clinical phase in the drug development process.

The demand for novel drugs to treat diseases, which are earlier considered easily curable is steadily enhancing and natural products continue to play a preliminary role in drug discovery, inviting researchers to embark on new chemical discoveries. Plant species from the Gentianaceae family are reported to be used as a traditional medicine in the treatment of diabetes [7-10]. Phytochemicals obtained from medicinal plant resources are always considered as an alternative bioactive lead molecule for the design and development of novel drugs [11-13]. *Exacum bicolor* Roxb. (Gentianaceae) is an endemic medicinal plant of western ghats, used as a folk lore medicine in treatment of diabetes, haemorrhage, colitis, fever, inflammation and skin burns [14-17]. Previous reports on the phytochemical investigations of the plant revealed the presence of secondary metabolites *viz.* glycosides, alkaloids, tannins, flavonoids, saponins, triterpenes and steroids [18-21].

In present study, through ADME analysis, *in silico* screening of the bioactive molecules is carried out and further the work has been extended to support the analysis through *in vitro* studies assessing for the antibacterial, antifungal, antioxidant and anticancer activities. The hexane and methanolic crude extracts of the aerial parts of *Exacum bicolor* were preliminarily tested for secondary metabolites and their presence

confirmed through UV and GC-MS analysis. The key objective of the current investigation is to recognize and classify the phytochemicals from *E. bicolor* through *in silico* screening for potential lead compounds aiding in the design and synthesis of newer drugs.

EXPERIMENTAL

The plant *Exacum bicolor* was collected from the grassland and shola forests of Bisele Ghat region of India (Sakaleshpura: 12º.79′68.70′′N, 75º.65′48.10′′E). The plant material *Exacum bicolor* (RRCBI-4009) was authenticated by the Regional Ayurveda Research Institute for Metabolic Disorders, (Central Council for Research in Ayurveda) Ministry of AYUSH, Government of India. All the chemicals and culture media used were procured from Hi Media and Merck Ltd., India, respectively.

Preparation of crude extract of plant: The aerial parts of the plant were air-dried, coarsely powdered (500 g) and was Soxhlet extracted successively with hexane and methanol. The crude extracts were concentrated under reduced pressure, dissolved in DMSO and stored in the refrigerator for further use.

Preliminary biochemical tests: Preliminary biochemical tests were carried out for the identification of presence/absence of phytoconstituents alkaloids, carbohydrates, flavonoids, phenolic compounds, tannins and proteins [22,23].

UV analysis of hexane and methanolic extracts of *Exacum bicolor*: The present study shows the partial characterization and identification of the phytocompounds from the hexane and methanol extracts of *E. bicolor* aerial parts by UV-Vis spectroscopy (Thermo-Fisher: GENESYS 10S UV-Vis v4.003 2L9Q082005 spectrophotometer). The peaks with maximum intensity and their wavelength (200-800nm) were recorded which helped in identifying the presence of possible phytochemicals [24].

GC-MS analysis of hexane and methanolic extracts of *Exacum bicolor*: Hexane and methanol crude extracts of *Exacum bicolor* were subjected to GC-MS analysis at Merieux NutriSciences Bangalore Pvt. Ltd. India. The sample (1 µL) was injected to the Agilent systems HP column-5MS of 30m length with an inner diameter of 0.25 mm and film 0.25 µm at 240 ºC. The carrier gas was helium with a flow rate of 1 mL/min. The MS (detector) was maintained at 230 ºC and the total run time was 55 min. The results were interpreted by comparing the spectra of unknown compounds with that of known compounds from National Institute Standard and Technology (NIST) (https://webbook.nist.gov/chemistry/) database. All the phytocompounds were further categorized to different classes based on their chemical properties referred from databases *viz*. Human Metabolome Database (HMDB) (https://hmdb.ca/metabolites), Pubchem (https://pubchem.ncbi.nlm.nih.gov/), ChEBI (https://www.ebi.ac.uk/chebi/init.do) and Chemspider (http://www.chemspider.com/).

ADME analysis: The key parameters considered were lipophillicity (log P) [25,26], molecular weight [27], bioavailability score [3,28], water solubility, gastrointestinal absorption, blood brain barrier permeability (BBB), drug likeliness (Lipinski's rule) and synthetic accessibility [1,25,29]. The permissible ranges for the parameters considered were referred from QikProp and Swiss ADME29 software manuals. A total of 32 phytochemicals were identified from the spectral analysis and these molecules were considered for ADME analysis by Swiss ADME software. The SMILES format of phytochemicals was retrieved from PubChem database and submitted to the Swiss ADME online software tool. The results obtained from Swiss ADME were analyzed to identify a phytochemical as lead compound.

Antimicrobial assay: Antimicrobial activity of the crude extracts was carried out by agar well diffusion method [30,31]. The test organisms, *viz*. *Bacillus pumilis* (MTCC 432), *Bacillus subtilis* (MTCC 441), *Streptococcus pyogens* (MTCC 2327), *Staphylococcus aureus* (MTCC 3160) (Gram-positive bacterial strains); *Corynebacterium diptheriae* (MTCC 637), *Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 424), *Klebsiella pneumonia* (MTCC 452) (Gram-negatuve bacterial strains) and fungal strains *Saccharomyces cerevisiae* (MTCC 783), *Aspergillus flavus* (MTCC 961), *Aspergillus fumigatus* (MTCC 969), *Aspergillus niger* (MTCC 953), *Candida albicans* (MTCC 854) were procure from the National Collection of Industrial Microorganisms laboratory, Pune, India.

Antioxidant assay: The aqueous and methanolic extracts from native and tissue cultured plants were evaluated for their antioxidant property by standard DPPH assay [32]. Ascorbic acid and gallic acid solutions were freshly prepared each time and considered as standard and positive control, respectively. The results obtained from DPPH assay was analyzed by calculating the percentage inhibition using the following formula:

$$\text{Inhibition (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Cytotoxicity assay: Cytotoxicity assay or cell viability assay was performed for the hexane and methanolic crude extracts of *E. bicolor*. THE MCF-7 breast cancer cell line and Normal MCF-10a were used to test the cell viability assay. DMEM (Dulbecco's modified Eagle's medium) supplemented with 4 mmol/1 L-glutamine, 4.5 g/L glucose and 10% heat-inactivated fetal calf serum was used to cultivate monolayer cell cultures. The 4×10^4 cells per well were seeded in 96-well plates with 100 µL of DMEM medium. Cells were incubated in a 5% CO_2 incubator for 24 h at 37 ºC. The crude extracts (0.04, 0.1, 0.2, 0.3, 0.6 and 1.2 mg/mL) were added to each well, respectively [33]. The culture medium was removed after 24 h and the cells were then washed with 100 µL of PBS. Cells that remained attached were fixed with 100 µL of 70% ethyl alcohol and incubated at room temperature for 1 h followed by the addition of methylene blue dye (100 µL) in order to remove ethyl alcohol. The plates were then incubated for 15 min at room temperature. A solution of 0.1 M HCl was used to elute the dye from the attached cells for 5 min at room temperature. The developed blue colour was measured at an absorbance of 630 nm using an microplate (Biorad) reader.

RESULTS AND DISCUSSION

Preliminary biochemical studies: Biochemical tests confirmed the presence of secondary metabolites like flavonoids, alkaloids, tannins, carbohydrates, *etc*. which might be respon-

TABLE-1
PRELIMINARY PHYTOCHEMICAL SCREENING OF HEXANE AND METHANOLIC EXTRACTS OF *E. bicolor*

	Alkaloids				Carbohydrates						Fixed oils and fats		Flavanoids		Phenolic compounds and tannins				Proteins		
	Mayers test	Dragendorff's test	Hagers test	Wagners test	Molischs test	Fehlings test	Barfoeds test	Benedicts test	Liebermanns test	Borntragers test	Oil stain test	Foam test	Filter paper test	Shinoda test	Ferric chloride test	Gelatin test	Lead acetate test	Bromine water test	Millons test	Biuret test	Ninhydrin test
Hexane extract	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	+
Methanolic extract	+	–	–	–	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+

sible for the excellent medicinal properties. The results are tabulated in Table-1.

UV-visible studies: UV-Visible spectroscopic analysis showed the presence of high intensity peaks (Fig. 1) in the wavelength range of 200 to 390 nm corresponding to the presence of anthocyanins and flavanoids (Table-2). Further, subjecting the extracts to GC-MS led to the identification of 99 compounds in the hexane and methanolic extracts of *E. bicolor* Roxb. (Fig. 2). Later, upon analyzing the GC-MS results using Pubchem, HMDB, ChEBI and Chemspider, 32 unique compounds with phytochemical potential were identified. Among these, 22 phytochemicals were from hexane extract and 10 were from methanolic extract (Table-3). These phytochemical compounds were considered for ADME analysis.

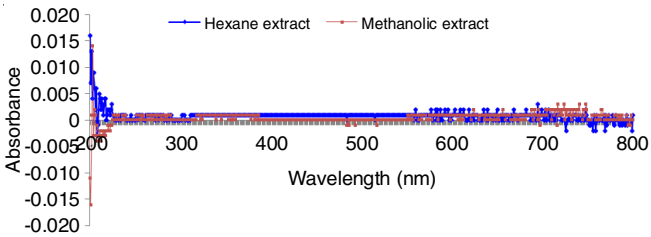

Fig. 1. UV-vis spectroscopy of methanolic and hexane extracts of *E. bicolor* Roxb

TABLE-2
UV-VIS SPECTROSCOPY SHOWING POSSIBLE PHYTOCHEMICALS PRESENT IN METHANOLIC AND HEXANE EXTRACTS OF *E. bicolor* Roxb.

	Hexane extract	Methanolic extract
Range	200-370	220-390
Highest peak	0.015	0.001
Number of peaks	58	45
Possible phytochemicals	Anthocyanins, flavonoids	Anthocyanins, flavonoids

ADME analysis: A detailed ADME analysis of all the 32 phytochemicals is given in Table-4.

Antimicrobial assay: The hexane and methanolic crude extracts of *Exacum bicolor* showed more prominent activity towards Gram-positive organisms with maximum towards *B. pumilis*, *B. subtilis* and *S. aureus*, very mild activity on *S. pyogenes*. For Gram-negative bacteria, maximum activity was displayed by *E. coli*, moderate activity with *P. aeuriginosa* and *K.*

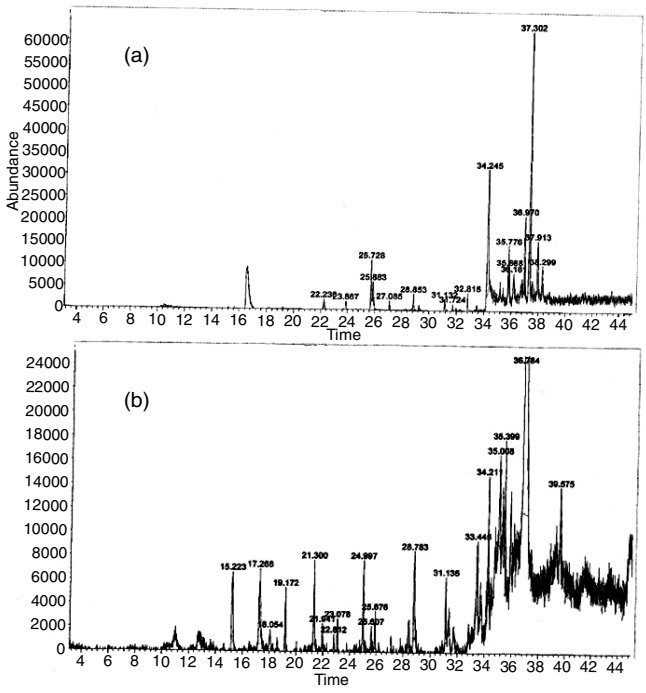

Fig. 2. GCMS graph of *E. bicolor* Roxb. extracts (a) hexane extract, (b) methanolic extract

pneumonia, almost no activity on *C. diphtheria*. The crude extracts of *E. bicolor* showed moderate activity towards *A. niger* and *A. fumigatus*, mild activity towards *A. flavus* and *C. albicans* and showed very mild or no activity against *S. cerevisiae*. The methanolic extract exhibited better activity than hexane extract and the activity is increased with the enhanced dose levels. The results are depicted in Table-5.

Antioxidant assay: The antioxidant property from hexane and methanolic extracts of *E. bicolor* were observed to be modest. The percentage inhibition of methanolic and hexane extracts was 73.26% and 62.34%, respectively.

Cytotoxicity assay: In order to investigate the synergistic effect of crude extracts, MCF-7 breast cancer cells were treated with different concentrations of crude extract (0.04, 0.10, 0.2, 0.3, 0.6, and 1.2 mg/mL) for 24 h. Controls included wells containing only cells and medium with and without 10% crude extract. The results are depicted in Fig. 3. A significant reduction in cell viability was observed as a function of concen-

TABLE-3
32 PHYTOCHEMICALS IDENTIFIED FROM HEXANE AND METHANOLIC EXTRACTS OF *E. bicolor* PLANTS

Compd. No.	Phytochemical	Pubchem id	Phytochemical class
	Hexane extract		
1	Sulphurous acid (2-ethyl hexyl hexyl ester)	6420784	Fatty acid ester
2	Sulfurous acid (2-ethyl hexyl isohexyl ester)	6420722	Fatty acid ester
3	Isopropyl myristate	8042	Fatty acid ester
4	Sulphurous acid, butyl octadecyl ester	11416806	Fatty acid ester
5	Geranyl isovalerate	5362830	Fatty alcohol
6	Diethyl phthalate	6781	Benzoic acid ester
7	Phthalic acid, isobutyl 2-methylpent-3-yl ester	91719720	Benzoic acid ester
8	Pyrimidine-2,4,6-(1*H*,3*H*,5*H*)-trione, 5-(3-(2-(4-tertbutylphenoxy)ethoxy)benzylidene	536531	Pyrimidine derivative
9	Bis(2-ethylhexyl)phthalate	8343	Benzoic acid ester
10	Hexdecanoic acid, methyl ester	117384	Fatty acid ester
11	1-Octanol, 2-butyl	19800	Fatty alcohol
12	Hydroxylamine *o*-decyl	34704	Fatty alcohol
13	2-Hexyl-1-octanol	545551	Fatty alcohol
14	Sulphurous acid, hexyl undecyl ester	6421372	Fatty acid ester
15	Sulphurous acid, pentyl undecyl ester	6421061	Fatty acid ester
16	Methoxyacetic acid, 4-tetradecyl ester	545726	Fatty acid ester
17	Methoxyacetic acid, 3-tridecyl ester	545726	Fatty acid ester
18	6-Tetradecanesulfonic acid, butyl ester	551402	Fatty acid ester
19	Cyclohexanol, 1-methyl-4-(1-methylethyl)	89437	Monoterpenoids
20	Phthalic acid, butyl dodecyl ester	96361	Benzoic acid esters
21	Heptadecane, 2,6,10,15-tetramethyl	41209	Sesquiterpenoids
22	Tetradecane, 2,6,10-trimethyl	85785	Sesquiterpenoids
	Methanolic extract		
23	*N,N*-Dimethyl-4-methoxy-3-methylphenethylamine	3064400	Phenethylamine derivate
24	Pyrimidine, 2-methoxy-5-methyl	580052	Pyrimidine derivative
25	Photocitral B	296248	Monoterpenoid
26	Phthalic acid, di((2-chlorocyclohexyl)methyl)ester	6423656	Benzoic acid ester
27	Nona-2,3-dienoic acid, ethyl ester	533672	Ester
28	Octadecanoic acid, 2-(2-hydroxyethoxy)ethyl ester	7788	Fatty acid ester
29	Chloromethyl 5-chloroundecanoate	543301	Ester
30	2-Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-, methyl ester	5357283	Ferulic acid ester
31	Erethrocentaurin	191120	Coumarin
32	1*H*-indole-2,3-dione, 1-methyl-, 3-hydrazone	600234	Indole derivative

TABLE-4
ADME PROPERTIES OF 32 PHYTOCHEMICALS IDENTIFIED FROM *Exacum bicolor*

Compd. No.	m.f.	m.w. (g/mol)	Lipophilicity logp (o/w)	Water solubility log S (Esol mol dm^{-3})	GI absorption	BBB permeable	Drug likeness (Lipinski)	Bioavailability score	Synthetic accessibility
1	$C_{14}H_{30}O_3S$	278.45	4.33	-4.27 Moderately soluble	High	Yes	Yes	0.55	4.12
2	$C_{14}H_{30}O_3S$	278.45	4.07	-4.16 Moderately soluble	High	Yes	Yes	0.55	4.12
3	$C_{17}H_{34}O_2$	270.45	5.53	-5.14 Moderately soluble	High	Yes	Yes (1 violation)	0.55	2.68
4	$C_{18}H_{39}NO_3S$	349.57	5.50	-5.74 Moderately soluble	Low	No	Yes	0.55	3.86
5	$C_{15}H_{26}O_2$	238.37	4.28	-4.13 Moderately soluble	High	Yes	Yes	0.55	3.04
6	$C_{12}H_{14}O_4$	222.24	2.29	-2.62 Soluble	High	Yes	Yes	0.55	1.93
7	$C_{18}H_{26}O_4$	306.40	4.20	-4.54 Moderately soluble	High	Yes	Yes	0.55	3.00
8	$C_{14}H_{14}N_2O_4$	274.27	1.29	-2.65 Soluble	High	No	Yes	0.55	2.49
9	$C_{24}H_{38}O_4$	390.56	6.17	-6.06 Poorly soluble	High	No	No	0.55	4.12
10	$C_{17}H_{34}O_2$	270.45	5.50	-5.48 Moderately soluble	High	Yes	No	0.56	2.87
11	$C_{12}H_{26}O$	186.33	3.82	-3.45 Soluble	High	Yes	Yes	0.55	2.30
12	$C_{10}H_{23}NO$	173.30	3.07	-3.07 Soluble	High	Yes	Yes	0.55	2.32
13	$C_{14}H_{30}O$	214.39	4.58	-4.17 Moderately soluble	High	Yes	Yes	0.55	2.07
14	$C_{17}H_{36}O_3S$	320.53	5.58	-5.47 Moderately soluble	High	No	Yes	0.55	4.20
15	$C_{16}H_{34}O_3S$	306.53	5.05	-5.11 Moderately soluble	High	No	Yes	0.55	4.35
16	$C_{17}H_{34}O_3$	286.45	4.91	-4.69 Moderately soluble	High	Yes	Yes	0.55	3.04
17	$C_{16}H_{32}O_3$	272.42	4.58	-4.39 Moderately soluble	High	Yes	Yes	0.55	3.38
18	$C_{18}H_{38}O_3S$	334.56	5.83	-5.43 Moderately soluble	Low	No	No	0.55	4.88

19	$C_{10}H_{20}O$	156.27	2.52	-2.37 Soluble	High	Yes	Yes	0.55	2.45
20	$C_{24}H_{38}O_4$	390.56	6.22	-5.57 Moderately soluble	High	No	Yes (1 violation)	0.55	3.41
21	$C_{21}H_{44}$	296.57	7.83	-7.33 Poorly soluble	Low	No	Yes (1 violation)	0.55	3.74
22	$C_{17}H_{36}$	240.47	6.51	-6.00 Moderately soluble	Low	No	Yes (1 violation)	0.55	3.09
23	$C_{12}H_{20}ClNO$	229.75	2.31	-3.41 Soluble	High	Yes	Yes	0.55	1.38
24	$C_6H_8N_2O$	124.14	0.96	-0.50 Very soluble	High	Yes	Yes	0.55	1.67
25	$C_{10}H_{16}O$	152.23	2.35	-2.24 Soluble	High	Yes	Yes	0.55	3.65
26	$C_{22}H_{28}Cl_2O_4$	427.36	5.25	-6.05 Poorly soluble	High	Yes	Yes (1 violation)	0.55	4.27
27	$C_{11}H_{18}O_2$	182.26	2.85	-2.37 Soluble	High	Yes	Yes	0.55	3.07
28	$C_{22}H_{44}O_4$	372.58	5.96	-5.59 Moderately soluble	High	No	Yes	0.55	3.78
29	$C_{12}H_{22}Cl_2O_2$	269.21	4.26	-3.92 Soluble	High	Yes	Yes	0.55	3.21
30	$C_{11}H_{12}O_4$	208.21	1.76	-2.32 Soluble	High	Yes	Yes	0.55	2.13
31	$C_{10}H_8O_3$	176.17	1.51	-2.01 Soluble	High	Yes	Yes	0.55	2.10
32	$C_9H_9N_{30}$	175.19	1.80	-3.04 Soluble	High	Yes	Yes	0.55	2.16

TABLE-5
ZONE OF INHIBITION VALUES FROM ANTIBACTERIAL AND ANTIFUNGAL ASSAY OF HEXANE EXTRACT (HE) AND METHANOLIC EXTRACT (ME) OF *E. bicolor* Roxb.

Test sample	Antibacterial activity								Antifungal activity				
	Gram-positive bacteria				Gram-negative bacteria								
	B.s	*B.p*	*S.a*	*S.p*	*K.p.*	*C.d*	*E.c*	*P.a*	*A. fumigatus*	*A. flavus*	*A. niger*	*C. albicans*	*S. cerevisiae*
H.E. (125 mg/mL)	07	08	07	–	08	–	10	09	09	–	08	–	–
H.E. (250 mg/mL)	08	10	08	–	09	–	11	09	11	–	09	–	06
H.E. (500 mg/mL)	09	11	11	08	10	07	13	11	12	07	11	–	06
M.E. (125 mg/mL)	11	14	09	07	11	–	11	12	12	07	11	09	07
M.E. (250 mg/mL)	14	16	13	07	11	–	14	13	13	08	12	09	07
M.E. (500 mg/mL)	16	17	16	08	12	08	15	14	14	11	13	11	08
Control (DMSO)	10	10	09	08	08	08	09	10	11	10	10	08	10
Antibiotic (ampicillin)	22	21	23	19	21	23	24	25	19	20	19	21	16

B.s = Bacillus subtilis, B.p = Bacillus pumilis, S.a = Staphylococcus aureus, S.p = Streptococcus pyogens, K.p = Klebsiella pneumoniae, C.d = Corynebacterium diptheriae E.c = Escherichia coli, P.a = Pseudomonas aeuriginosa

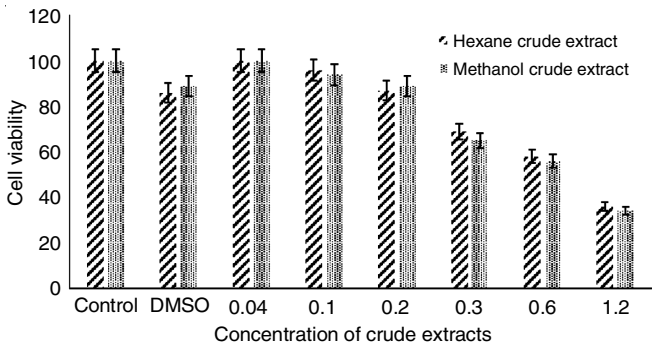

Fig. 3. MCF-7 cancer cell line was incubated with various concentrations of the crude extracts (0.04, 0.10, 0.15, 0.20, 0.3, 0.6, and 1.2 mg/mL) for 24 h

tration. The methanolic extracts exhibited better activity and the reduction in the cell viability was found to enhance with increased concentrations.

The results obtained from ADME analysis revealed amongst the 32 phytochemicals studied for ADME properties only two phytocompounds *viz*. phthalic acid butyl dodecyl ester and pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione-5-(3-(2-(4-tertbutylphenoxy)ethoxy)benzylidene satisfied all the conditions and had the parameter values within the acceptable range. The two chemical constituents could serve as lead moieties. The antimicrobial activity of the crude extracts displayed the results similar to the earlier works on related plant species by several researchers [18,19,34]. With respect to antioxidant properties, the methanolic extracts showed better activity when compared to hexane extracts and the results are on par with Appaji *et al*. [20]. The cytotoxicity assay displayed moderate activity in reducing the cell viability and there were no earlier reports of cell viability assays on the *E. bicolor* extracts. Although the crude extracts displayed moderate antimicrobial, antioxidant and cytotoxic properties, the *E. bicolor* extracts still consider as the principal antidiabetic source as claimed by the ethnomedical societies and Ayurveda [35,36].

Conclusion

This study enlisted the ADME properties of the phytochemicals observed in *Exacum bicolor* using the Swiss ADME. Only two molecules *i.e*. phthalic acid butyl dodecyl ester and pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione-5-(3-(2-(4-tertbutylphenoxy)ethoxy)benzylidene were identified as possible drug candidates amongst, a total of 32 molecules identified from the crude extracts of *E. bicolor*. Since *in silico* approaches are based on models and algorithms, an experimental validation must be carried out for further authentication of drugs 5. The *in silico* screening of the bioactive molecules sharing the global

platform in the present study surpasses the conventional methods *viz*. extraction, isolation and purification of compounds with relative ease and less expenditure. Further, the pharmacological properties of only these two molecules can be evaluated for confirming their therapeutic abilities instead of entire secondary metabolite analysis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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