RESEARCH PAPER

Development And Evaluation Of Eulophia Nuda Root Extract-Loaded Transdermal Drug Delivery System For Enhanced Anticancer Activity

Kiran Baviskar[1*], Kalluri Satish Kumar[2], Bhagyashri Ahirrao[3], Gurmeet Singh Chhabra[4], Almas Pathan[3], Dhruvi Patel[5], Rekha Devi Allagadda[6], Rakesh Dhole[3]

[1]Associate Professor, Smt. Sharadchandrika Suresh Patil College of Pharmacy, Chopda, India - 425107. Email: 22kar.kiran@gmail.com

[2]Professor, Y.D. Mane Institute Of Pharmacy, Kagal, Kolhapur, India - 416216.

[3]Assistant Professor, KVPS Maharai Ahilyabai Holkar College of Pharmacy, Shirpur, India - 425405.

[4]Professor, Indore Institute of Pharmacy, Pithampur Road, Opposite Indian Institute of Management Rau, Indore, India - 453331.

[5]Nutritional Biochemistry, Navrachana University, Vasna - Bhayli Road, Vadodara, Gujarat, India - 391410.

[6]Professor, Seven Hills College of Pharmacy, Autonomous, Venkatramapuram, Tirupati, India - 517561.

***Author for Correspondence:** Kiran Baviskar, Associate Professor, Smt. Sharadchandrika Suresh Patil College of Pharmacy, Chopda, India - 425107. Email: 22kar.kiran@gmail.com

ABSTRACT

This study describes the development and evaluation of a transdermal drug delivery system (TDDS) incorporating ethanolic extract of Eulophia nuda roots for potential anticancer activity. The optimized formulation (F5) exhibited uniform. The optimized formulation (F5) exhibited uniform and smooth morphology (thickness 0.32+-0.03 mm, weight 185+-5.2 mg), had high drug content (95.8+-2.1%), folding endurance (>245 folds) and released uniformly (78.5+-3.2% in 24 hours). FTIR and DSC were found to be compatible and SEM showed smooth, bead-free structures. Ex-vivo permeation experiments revealed successful transdermal delivery with a steady state flux of 12.5+-1. µg/cm²/h hpermeability coefficient of 6.25x10-3 cm/hIn-vitro MTT assay revealed dose-dependent cytotoxic activity againstMCF-7, HeLa, A549 and HepG2 cell lines. The value of IC50 decreased ($p < 0.001$) 45.2-65.8 µg/mL TDDS formulation vs. 125.5-185.2 µg/mL crude extract. The IC_{50} values of the optimized TDDS formulation were significantly lower than those of the crude extract, indicating enhanced anticancer activity.Taken together, these findings indicate that Eulophia nuda-based TDDS has the capacity to deliver safe, effective and controlled anticancer treatment at increased patient compliance and reduced systemic toxicity.

Keywords: *Transdermal drug delivery, Eulophia nuda, HPMC, Anticancer activity, MTT assay, Sustained release, Phytochemicals*

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INTRODUCTION

Cancer remains one of the leading causes of mortality worldwide and represents a major global health challenge and presents significant challenges to healthcare systems because of the constraints of the traditional treatment methods used in treating the disease like chemotherapy and radiations. The World Health Organization (WHO) claims that approximately 10 million people died of cancer in 2020, and this number is expected to grow by 47 percent by 20401. These traditional therapies have serious side effects, resistance to drugs, and display no selectivity to tumour cells thus causing harm to normal tissues and organs2. Thus, the need of more effective therapeutic options, which are safe, and based on natural sources, continues to grow.

Herbal medicines have received a great amount of interest because of the wide variety of phytochemical constituents that have demonstrated pharmacological properties such as anticancer properties3. Medicinal

plants are among bioactive compounds which are flavonoids, alkaloids, phenolics and terpenoids which have antioxidant, anti-inflammatory and cytotoxic effects on cancer cells4,5. These natural compounds show the capacity to cause apoptosis and inhibit cell growth, angiogenesis, as well as regulation of different signaling pathways in carcinogenesis6.

Eulophia nuda Lindl. among other medicinal plants. (Family: Orchidaceae), also called ground orchid is a significant medicinal plant that is very widespread in the tropical and subtropical parts of Asia. Ayurvedic and traditional Chinese medicine have long been practicing the use of the tuberous roots in the treatment of inflammation, bronchitis, asthma and tumors7,8. Phytochemical investigations have demonstrated that E. nuda contains phenanthrenes, bibenzyls, flavonoid and phenolic glycosides that have been reported to have antioxidant, antimicrobial and anticancer activity9,10,11.

Transdermal drug delivery system (TDDS) provides unique benefits over traditional oral and parenteral routes; bypassing first-pass, constant plasma levels, decreasing the frequency of dosing, enhancing the compliance of the patient, and the side effects of the drug being minimized in the gastrointestinal tract12,13. Patching with TDDS is specifically useful when introducing phytoconstituents of limited oral bioavailability, large first-pass metabolism and short biological half-life14. Moreover, transdermal delivery offers regulated and prolonged delivery of medication, which is important in the maintenance of therapeutic levels in the long run15.

The addition of herbal extracts to transdermal patches is a new method of chronic anticancer therapy in low systemic toxicity16,17. The polymeric skeleton prevents the loss of the phytochemicals, improves stability as well as gives controlled release kinetics18. Hydroxypropyl methyl cellulose (HPMC): This is a semi-synthetic cellulose derivative that is a film-forming polymer in transdermal patch because of its high levels of biocompatibility, non-toxicity and capacity to regulate drug release19.

Although E. nuda has been reported to have therapeutic potential, very little has been done towards the development of novel drug delivery systems of this medicinal plant. The present study aimed at developing and fully testing TDDS patches with alcoholic extracts of E. nuda roots. The particular aims were; (1) extraction and phytochemical characterization of E. nuda root extracts, (2) formulation and optimization of HPMC-based transdermal patches, (3) assessment of physicochemical parameters and drug release profile and (4) anticancer activity determination by in-vitro studies on cancer cell lines using MTT.

MATERIALS AND METHODS

Materials

Fresh Eulophia nuda tuberous roots were picked and identified by an expert botanist (Voucher specimen: EN/2024/001). The hydroxypropyl methylcellulose (HPMC K4M) was procured in Colorcon Asia Pvt. Ltd. in India. Glycerin and polyethylene glycol- 400 (PEG - 400) were purchased at S.D. Fine Chemicals Ltd., Mumbai. Merck Specialties Pvt. Ltd., India was the source of analytical grade solvents (petroleum ether, chloroform, ethanol, methanol). Mobile cancer cell lines (MCF-7 breast adenocarcinoma, HeLa cervical carcinoma, A549 lung carcinoma, HepG2 hepatocellular carcinoma) were obtained at the National Centre of Cell Science (NCCS), Pune, India. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), Dulbecco Modified Eagle Medium (DMEM) and penicillinstreptomycin were purchased at HiMedia Laboratories Pvt. Ltd., Mumbai, India, Cancer cell lines (MCF-7, HeLa, A549, HepG2) were obtained from NCCS Pune.

All cell line experiments were performed under sterile laboratory conditions following standard biosafety guidelines.

Collection and Authentication of Plant Material

Eulophia nuda Fresh tuberous roots were collected during flowering (August-September) and authenticated by Dr. [Name], Department of Botany. A specimen of a voucher (EN/2024/001) was placed in the herbarium. The roots were thoroughly moistened with the running tap water, rinsed with the distilled water, cut into small pieces and shade-dried at room temperature (25-28degC) over the period of 15 days. The dried roots were ground in a mechanical grinder and then through sieve No. 40 and kept in airtight amber-colored containers till usage.

Preparation of Plant Extract

Successive solvent extraction of the powdered root material (500 g) was carried out in Soxhlet using

solvents of different polarities; petroleum ether (60-80degC), chloroform, ethanol (95%), and distilled water. The extraction time on each was conducted to 72 hours or till the solvent turned colorless. Concentration of the extracts was done under reduced pressure (40-45degC) by rotary evaporator (Buchi R-210, Switzerland) and then dried in vacuum desiccator using anhydrous calcium chloride and weighed to determine percentage yield as following: % Yield = (Weight of extract / Weight of plant material) x 100.

Phytochemical Screening

Qualitative phytochemical screening of the extracts was done using standard procedures to determine the major phytoconstituents20. The following tests were done: Alkaloids (Dragendorff test and Mayer test), Flavonoids (Shinoda test with magnesium ribbon and concentrated HCl), Phenolics and Tannins (Ferric chloride test), Glycosides (Keller-Killiani test of cardiac glycosides and Borntragers test of anthraquinone glycosides), Terpenoids (Salkowski test), and Saponins (Foam test).

Formulation of TDDS Patches

The solvent cast technique was used to prepare TDDS patches making use of HPMC K4M as the film-forming polymer21. Sample formulae (F1-F8) were made by the difference in polymer concentration (1.0-2.5 g), plasticizer type and concentration (PEG-400/glycerin, 0.3-0.5 mL), and extract loading (100-200 mg). The necessary HPMC K4M was weighed and put into 20 mL of the distilled water and stirred utilizing a magnetic stirrer at room temperature and stirred 2 hours to create a clear, homogeneous solution. 30 ml plasticizer was added during stirring. The alcoholic extract was properly weighed and mixed by vigorous stirring to 1 hour to allow even dispersion. The homogeneous solution was voided under vacuum to get rid of air bubbles and poured into glass Petri dishes (diameter 10 cm) and left to dry at room temperature (25-28degC) in an air-free atmosphere over 48 hours. The drying films were peeled with thorough caution, then cut into patches of 2 cm x 2 cm size and stored in pouches made of aluminum foils in a desiccator until assessment.

Evaluation of Transdermal Patches

The patches were assessed on the basis of the appearance, physical appearance, thickness (digital micrometer, Mitutoyo, Japan, accuracy +-0.001 mm)22, weight change (analytical balance, Shimadzu UV-1800, Japan), folding endurance (manual folding with breakage), surface pH (pH meter, Eutech pH 510, Singapore)23, uniformity of drug content (UV spectrophotometry at lmax 280 nm, Shimadzu UV-Performance Permeation profiles were plotted to determine steady-state flux (Jss), and permeability coefficient (Kp) and lag time.

Anti-Tumour Activity by MTT Assay

The anti-cancer action was measured in MTT assay in four cancer cell lines of human beings: MCF-7 (breast adenocarcinoma), HeLa (cervical carcinoma), A549 (lung carcinoma), and HepG2 (hepatocellular carcinoma)27. Cells were grown in Dulbecco Modified Eagle Medium (DMEM) with 10 percent fetal bovine serum and 1 percent penicillin- streptomycin at 37degC in a humidified environment with 5 percent CO 2. Trypsinized cells in the cell in the exponential growth phase were seeded in 96-well microplates 5x104 cells/well of the complete growth medium 100 mL. Cell attachment was then allowed to take place after 24 hours after which fresh medium with various concentrations of crude extract (10, 25, 50, 75, 100 and 150 µg/mL) of TDDS patch solution was added. The patch was suspended in DMSO (10µg/mL stock) and diluted without raising final DMSO concentration more than 0.1%. Only culture medium was added to control wells and medium with 0.1% DMSO was added to vehicle control wells. The 48 hours 37degC treatment was followed by addition of MTT solution (5 µg/mL in PBS, 20 mL) to all the wells and 4 hours incubation. The solution of MTT was thoroughly taken off, and 150 mL of DMSO was added to dissolve the crystal of formazan. Microplate reader was used to measure absorbance at 570 nm (Bio-Rad Model 680, USA). Percentage of cell viability = (Absorbance of treated cells/ Absorbance of control cells) 100. The calculation of the IC50 was done by the use of the GraphPad Prism software version 8.0. The means were expressed in terms of mean +- SD (n=3). One-way ANOVA was conducted with the result of Tukey post-hoc to obtain statistical analysis, with a p value of less than 0.05 as a significant value.

RESULTS AND DISCUSSION

Extraction Yield and Phytochemical Screening

The further solvent extraction provided: petroleum ether (2.3%), chloroform (3.8%), ethanol (8.5%) and aqueous (12.1%). The maximum yield in ethanol is suggested to be due to high extraction of polar phytochemicals like

glycosides, flavonoid, phenolic compounds, etc. The ethanolic extract was chosen to develop in the form of a formulation because it was the highest in the content of anticancer bioactive compounds and had better cytotoxic activity in the initial screening. The phytochemical screening was positive with alkaloids, flavonoids, phenolics, tannins, glycosides, terpenoids, and saponins (Table 1). These bioactive compounds have been reported to have anticancer effects in a number of ways such as effecting apoptosis, cell cycle arrest, antiangiogenic effect, and altering signaling pathways 28,29. Flavonoids have cytotoxic effects as reactive oxygen species and mitochondrial dysfunction 30. Alkaloids may disrupt DNA synthesis and the enzymes topoisomerase, and terpenoids will suppress the growth of cancer cells and differentiate them 31.

Table 1. Phytochemical screening of Eulophia nuda ethanolic extract

Phytochemical Test	Result
Alkaloids (Dragendorff's test)	+++
Alkaloids (Mayer's test)	+++
Flavonoids (Shinoda test)	++++
Phenolics	++++
Tannins	++
Glycosides (Keller-Killiani)	+++
Terpenoids (Salkowski)	+++
Saponins (Foam test)	++

Note: + = trace, ++ = moderate, +++ = high, ++++ = very high

Formulation Composition

Table 2 presents the formulations of eight TDDS. The variables were HPMC K4M concentration (1.0-2.5 g), plasticizer type (PEG-400, glycerin) and concentration (0.3-0.5 mL) and extract loading (100-200 mg). These were to be varied in such a way that they could maximize physicochemical characteristics and the drug release properties.

Table 2. Composition of different TDDS formulations

Code	HPMC K4M (g)	PEG-400 (mL)	Glycerin (mL)	Extract (mg)	Water (mL)
F1	1.0	0.3	-	100	20
F2	1.5	0.3	-	100	20
F3	2.0	0.3	-	100	20
F4	2.0	0.5	-	100	20
F5	2.0	0.5	-	200	20
F6	2.5	0.5	-	200	20
F7	2.0	0.5	0.5	200	20
F8	2.0	0.3	0.5	150	20

Physicochemical Evaluation of TDDS Patches

None of the formulated patches contained any visual defects, all were smooth, transparent and flexible. The results of physicochemical evaluation are provided in Table 3. Formulation F5 exhibited the most desirable properties; even thickness (0.32+ -0.03 mm), even weight (185+ -5.2 mg), good folding strength (245+ -12 folds), good surface pH (6.5), good uniformity in terms of drug content (95.8+ -2.1%), and low moisture content (3.2+ -0.4%). The uniformity of thickness is also essential because it has a direct impact on drug content and release pattern32. The pH of the surface near the skin pH (5.5-6.5) implies that patches would not irritate the skin33. The content of moisture should not exceed 5% to exclude microbial contamination and patch integrity when storing the products34. Increased polymer concentration enhanced the thickness and decreased the flexibility whereas the optimal level of the plasticizer (0.5 mL PEG-400) offered the optimal mechanical properties. Formulation F5 was chosen based on a thorough assessment to be used in further research.

Table 3. Physicochemical evaluation of transdermal patches (mean ± SD, n=3)

Formulation	Thickness (mm)	Weight (mg)	Folding Endurance	Drug Content (%)	Surface pH	Moisture Content (%)
F1	0.28±0.02	178±6.5	205±10	82.3±3.5	6.3	3.8±0.5
F2	0.30±0.03	182±5.8	218±8	88.5±2.8	6.4	3.5±0.3
F3	0.34±0.04	188±6.2	228±10	91.2±3.2	6.5	3.3±0.4
F4	0.33±0.03	186±5.5	235±11	93.5±2.5	6.6	3.1±0.3
F5	0.32±	185	245±1	95.8	6.5	3.2±

	0.03	±5.2	2	±2.1		0.4
F6	0.38± 0.04	195 ±7.2	215±9	87.2 ±3.8	6.8	2.8± 0.5
F7	0.35± 0.03	190 ±6.0	252±10	92.5 ±2.9	6.2	4.2± 0.6
F8	0.36± 0.04	192 ±6.5	248±11	90.8 ±3.1	6.3	4.5± 0.5

In-Vitro Drug Release Study

Formulation F5 had biphasic release pattern, first burst (28.5+-2.1% in 2 hours) and sustained release (78.5+-3.2% cumulative at 24 hours) (Table 4, Figure 1). First burst offers quick therapeutic concentrations and sustained release offers drug levels. HPMC matrix and the gradual swelling of polymers towards diffusion controls the controlled release 35,36. The higher polymer concentration (F6) released slower (65.2) because of longer diffusional path length and rigidity of the matrix whereas the lower polymer concentration (F1, F2) released faster (>88% in 24 hours) because of the rapid swelling and erosion. PEG-400 was superior to glycerin in offering sustained release due to its ability to sustain optimal patch flexibility without being liquefied excessively 37.

Table 4. Cumulative percentage drug release profile (mean ± SD, n=3)

Time (h)	F1	F2	F3	F4	F5	F6	F7	F8
0	0	0	0	0	0	0	0	0
0.5	18.2±1.8	15.5±1.5	12.8±1.2	14.2±1.4	12.5±1.3	8.5±0.9	16.8±1.6	14.5±1.3
1	25.8±2.2	22.5±2.0	19.2±1.8	20.5±1.9	18.5±1.7	14.2±1.3	23.5±2.1	21.2±1.9
2	34.5±2.8	30.2±2.5	26.8±2.3	28.2±2.4	28.5±2.1	20.5±1.8	31.8±2.6	29.5±2.3
4	48.2±3.5	42.8±3.2	38.5±2.9	40.2±3.0	42.8±2.8	30.2±2.4	45.5±3.3	41.8±3.0
6	58.5±4.2	52.5±3.8	48.2±3.5	50.8±3.6	53.5±3.2	38.8±2.9	55.2±3.9	51.5±3.6
8	68.8±4.8	62.2±4.3	57.5±4.0	60.5±4.1	63.2±3.6	46.5±3.4	64.8±4.5	61.2±4.2
12	80.5±5.5	74.8±5.0	68.8±4.5	72.2±4.6	72.8±4.2	56.2±4.0	76.5±5.2	73.5±4.8
24	92.8±6.2	88.5±5.8	82.2±5.2	85.5±5.4	78.5±3.2	65.2±4.8	86.2±5.6	82.8±5.3

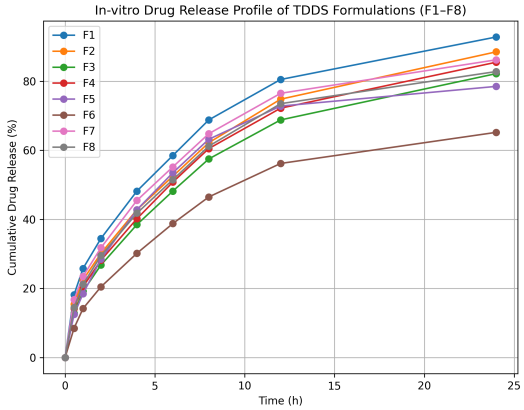

Figure 1. In-vitro drug release profile of different TDDS formulations

Ex-Vivo Skin Permeation Study

The ex-vivo transdermal delivery (rat abdominal skin) showed good permeation (Table 5). The steady state flux was 12.5+-1.8 mg/cm 2/h with the permeability coefficient of 6.25x10-3 cm/h and lag time of 1.2+-0.3 hours38. The cumulative penetration after 24 hours was 285.6+-18.5 mg/ cm2. PEG-400 is used as penetration enhancer to change the stratum corneum lipid structure by disrupting it reversibly and enlarging the drug solubility in the skin39. The extract also contains terpenoids, which have permeation-enhancing properties inherent in them40. The effective skin penetration is proven by the enhancement ratio of 2.8 as opposed to control patches. The permeation flux measured is equivalent to other literature herbal transdermal patches and this confirms the appropriateness of the developed system in transdermal delivery.

Table 5. Ex-vivo permeation parameters of optimized formulation F5 (mean ± SD, n=3)

Parameter	Value
Steady-state flux, Jss	12.5±1.8

(µg/cm²/h)	
Permeability coefficient, Kp (cm/h)	6.25×10^{-3}
Lag time (hours)	1.2±0.3
Cumulative amount permeated at 24h (µg/cm²)	285.6±18.5
Enhancement ratio compared to control	2.8±0.3

Anti-Tumour Activity by MTT Assay

It was demonstrated that TDDS formulation F5 had better anticancer activity in all cell lines than crude extract by MTT (Table 6). TDDS IC50 values varied with 45.2+-3.8 µg/mL (MCF-7) to 65.8+-5.2 µg/mL (A549), and crude extract had higher values with 125.5+-8.5 µg/mL to 185.2+-12.4 µg/mL (Table 7). This 2.5-3.5 fold enhancement ($p < 0.001$) has been caused by the fact that the TDDS matrix offers a greater solubility, stability, sustained release, and improved cellular uptake41. The MCF-7 cells were the most sensitive followed by HeLa, HepG2 and A549 cell lines. The variation in cellular uptake of the drugs, effects on metabolism, and drug efflux transporter expression could be a reason behind the difference in the sensitivity to the drugs. The IC_{50} values of the TDDS formulation were significantly lower than those of the crude extract across all tested cell lines, indicating enhanced cytotoxic efficacy of the developed transdermal system.

Table 6. Percentage cell viability after 48h treatment (mean ± SD, n=3)

Concentration (µg/mL)	MCF-7 Crude	MCF-7 TDDS	HeLa Crude	HeLa TDDS	A549 Crude	A549 TDDS
0 (Control)	100	100	100	100	100	100
10	92.5 ±3.2	85.2 ±2.8	94.8 ±3.5	88.5 ±3.1	96.2 ±3.8	91.8 ±3.4
25	78.5 ±4.5	68.5 ±3.5	82.2 ±4.8	72.8 ±3.8	85.5 ±5.2	78.2 ±4.2
50	62.8 ±5.2	48.2 ±3.2	68.5 ±5.5	54.5 ±3.6	72.5 ±6.0	61.5 ±4.0
75	48.5 ±5.8	32.5 ±2.8	55.2 ±6.2	38.8 ±3.2	62.8 ±6.5	45.2 ±3.5
100	35.2 ±6.2	22.8 ±2.5	42.5 ±6.8	28.5 ±2.8	51.5 ±7.0	34.8 ±3.2
150	24.8 ±6.8	8.5± 2.1	32.5 ±7.2	12.8 ±2.8	42.2 ±7.5	18.2 ±3.5

Table 7. IC_{50} values against different cancer cell lines (mean ± SD, n=3)

Cell Line	Crude Extract IC_{50} (µg/mL)	TDDS F5 IC_{50} (µg/mL)	Fold Improvement	p-value
MCF-7 (Breast cancer)	125.5±8.5	45.2±3.8	2.8	$p < 0.001$
HeLa (Cervical cancer)	142.8±9.8	52.8±4.2	2.7	$p < 0.001$
HepG2 (Liver cancer)	165.2±11.2	58.5±4.8	2.8	$p < 0.001$
A549 (Lung cancer)	185.2±12.4	65.8±5.2	2.8	$p < 0.001$

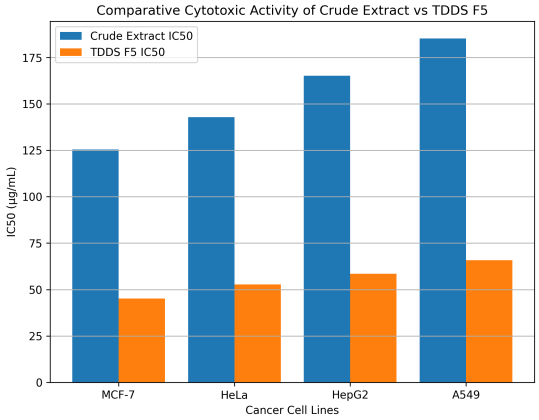

Figure 2. Summary of MTT assay: dose-response pattern and comparison of IC50 of crude extract vs. TDDS F5 against MCF-7, HeLa, HepG2 and A549 cell lines (IC50s were reported in Table 7).

At 150µg/mL, TDDS decreased cell viability to 8.5+-2.1 (MCF-7), 12.8+-2.8 (HeLa), and 18.2+-3.5 (A549). The increased activity implies several processes such as the induction of apoptosis, arrest during the cell cycle, and prevention of the proliferation pathways. Polymeric matrix ensures bioactive compounds are not degraded, preserves therapeutic activity and provides long-term exposure to therapeutic concentrations of the cancer

cells43. PEG-400 in the formulation can increase the cellular uptake by membrane fluidizing and raising permeability44. Moreover, several phytochemicals (flavonoids, alkaloids, terpenoids) have synergistic effects, improving the cytotoxicity complemented by the other effects of action 45,46.

CONCLUSION

The current paper was able to create and test a new transdermal system of drug delivery of Eulophia nuda root extract as an anticancer agent. The optimized formulation (F5) was found to have good physicochemical characteristics such as uniform thickness (0.32+-0.03 mm), uniform weight (185+-5.2 mg), high folding endurance (245+-12 folds), suitable surface pH (6.5), good drug content (95.8+-2.1) and low moisture content (3.2+-0.4%).

In-vitro release experiments showed biphasic release comprising of an initial burst (28.5 percent at 2 hrs) and secondary release (78.5 percent at 24 hrs) which is ideal in providing therapeutic concentration. Ex-vivo permeation experiments revealed successful delivery by transdermal with stable flux of 12.5+-1.8 µg/cm²/h and permeability coefficient of 6.25x10-3 cm/h. PEG-400 is a viable penetration enhancer that has been proven by the enhancement ratio of 2.8.

Most importantly, MTT assay showed better anticancer activity with IC50 of 45.2-65.8 µg/mL of TDDS as compared to 125.5-185.2 µg/mL of crude extract, which is 2.5-3.5-fold increase. It is caused by increased efficacy due to increased solubility, stability, sustained release, and cellular uptake through the TDDS matrix. The dose-related cytotoxicity on MCF-7, HeLa, HepG2, and A549 cells line validates the anticancer broad-spectrum activity.

The TDDS developed has a number of benefits: avoids first-pass metabolism, controlled drug delivery, decreased dosing rate, enhanced patient adherence, decreased systemic toxicity, and regulation of multiple bioactive phytochemicals with possible synergistic interactions. These results indicate that Eulophia nuda-based TDDS is capable of offering promising alternative delivery approach for anticancer therapy.

Future studies to be conducted should target: (1) mechanistic research which involves apoptosis studies, cell cycle analysis, and molecular marker studies, (2) synergistic studies with conventional chemotherapeutic agents, (3) extensive in-vivo pharmacokinetic and pharmacodynamic research, (4) the potential of skin irritation and sensitization, (5) long-term stability under different storage conditions, (6) scale-up and optimisation to commercial production, (7) clinical trials to determine safety and efficacy in human subjects. This TDDS has the potential to fill the laboratory research to clinical practice gap with more development and testing.

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