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RESEARCH PAPER

CRYPTOLEPINE, A PLANT-DERIVED ALKALOID, DIFFERENTIALLY REGULATES SIGNAL TRANSDUCTION PATHWAYS IN HUMAN EMBRYONIC KIDNEY (HEK293) CELLS

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ABSTRACT

Cryptolepine is the main alkaloid in the medicinal plant Cryptolepis sanguinolenta. This plant-derived alkaloid has innumerable pharmaco-biological properties, including anti-microbial, anti-hyperglycaemic and anti-inflammatory in diverse in vitro and in vivo systems. We have previously shown that cryptolepine differentially regulates signalling pathways in human hepatoma (HepG2) cells. Hence, this current study aimed to investigate the effects of cryptolepine on these pathways in human embryonic kidney (HEK293) cells to ascertain whether what we reported in the HepG2 cells is cell dependent. The Cignal Finder Multi-Pathway Reporter Array was used to screen the effects of cryptolepine on the pathways in the HEK293 cells. Next, some genes in the differentially regulated pathways were assessed using RT-qPCR. Cryptolepine up-regulated 9 pathways, including p53, IRF1 and PR, supported by increased IRF1 and PR transcripts. Contrarily, cryptolepine down-regulated 27 pathways, including STAT3, c-Myc and HIF-1α, bolstered by decreased HIF1-α and STAT3 transcripts. The regulations of the pathways in the HEK293 cells differed from those observed in the HepG2 cells. This study revealed that cryptolepine differentially regulates signalling pathways and regulates these pathways differently in diverse cells. The results from our studies support the pharmaco-biological effects of cryptolepine in different cells.

Keywords: Cryptolepine, Cell Signalling, Progesterone Receptor, IRF1, STAT3, HIF-1α

INTRODUCTION

Cryptolepine is the main alkaloid in the West and Central African medicinal plant *Cryptolepis sanguinolenta*. This plant-derived alkaloid has been widely reported to exhibit numerous pharmacological and biological properties, including antimalarial (Kirby *et al.*, 1995), anti-bacterial (Gibbons *et al.*, 2003), anti-fungal (Sawer *et al.*, 1995) and anti-hyperglycaemic (Oyekan *et al.*, 1988; Bierer *et al.*, 1998) effects under different *in vitro* and *in vivo* conditions. In addition to these many pharmaco-biological effects, cryptolepine has also been reported to have anti-inflammatory activity in various animal model systems (Olajide *et al.*, 2009; Olajide *et al.*, 2013a). Cryptolepine has been reported to exhibit its anti-inflammatory effects by targeting inflammatory mediators and promoters such as the prostaglandin E_2 (PGE_2), cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS) and nuclear factor kappa B (NF-κB) pathways (Olajide *et al.*, 2010; Olajide *et al.*, 2013a, 2013b).

Some researchers believe that cryptolepine has anti-cancer effects due to its anti-inflammatory and cytotoxic properties. Cryptolepine has been reported to inhibit the growth of cancer cells by inhibiting topoisomerase II and inducing DNA damage and the p53 signalling pathway (Zhu & Gooderham, 2006; Pal & Katiyar, 2016). Also, cryptolepine has been reported to hamper cancer cell growth by inhibiting the mTOR signalling (Pal *et al.*, 2017). But interestingly, cryptolepine (at 7.5 µM) has been shown to selectively inhibit melanoma cells compared to normal melanocytes after 48 h (Pal *et al.*, 2017). This finding implies that at lower concentrations, cryptolepine is selectively toxic to melanoma cells, which makes cryptolepine a potential agent in managing melanoma and other cancers.

Persistent hyperactivation of the interleukin-6/signal transducer and activator of transcription 3 (IL-6/STAT3) signalling pathway leads to angiogenesis (Gao *et al.*, 2017), stemness (Yao *et al.*, 2016) and epithelial-mesenchymal transition (Liu *et al.*, 2015) in the tumour microenvironment. Moreover, aberrant induction of IL-6 is detrimental to the hepatocytes and could lead to the development of hepatocellular carcinoma (Zhou *et al.*, 2017; Gai *et al.*, 2020). Hence, targeting the IL-6/STAT3 signalling could be a novel approach to preventing or treating cancers because phospho-STAT3 contributes to malignant transformation and progression (Johnson *et al*., 2018). Interestingly, we have shown that cryptolepine inhibits the IL-6/STAT3 pathway in human hepatoma cells by inhibiting STAT3 phosphorylation and reducing IL-23 levels (Domfeh *et al.*, 2021). This finding implies that cryptolepine-containing remedies such as *C. sanguinolenta* extract could be repurposed for managing liver and other cancers.

The functions of type 1 interferon (IFN-1) signalling pathway in anti-viral and anti-cancer immunosurveillance are well-known (reviewed in Fuertes *et al.*, 2013). Recombinant IFNs have been clinically approved and have successfully been used in treating cancers and viral infections due to the biological effects of IFNs (Bekisz *et al.*, 2010; Chi *et al.*, 2017). We have demonstrated that cryptolepine activates the IFN-1 response pathway in THP1-derived macrophages and human hepatoma (HepG2) and human embryonic kidney (HEK293) cells (Domfeh *et al.*, 2022). This finding implies persons exposed to cryptolepine-containing herbal preparations such as *C. sanguinolenta* extract will be fortified and, consequently, protected against infectious diseases and cancers.

We have reported that cryptolepine differentially regulates 45 signalling pathways in HepG2 cells. Cryptolepine up-regulated 17 signalling pathways and down-regulated 12 (Domfeh *et al.*, 2021). Hence, this current study aimed to investigate the effects of

cryptolepine on these signalling pathways in HEK293 cells to ascertain whether what we reported in the HepG2 cells is cell dependent. The significance of the current study is that signalling pathways differentially regulated by cryptolepine in HEK293 cells would be identified. The data obtained would enhance research on the effects of cryptolepine in different biological systems.

MATERIALS AND METHODS

Reagents and chemicals

The cryptolepine used in the study was donated by Prof. Kwesi Boadu Mensah, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana. How the cryptolepine was isolated and purified from the roots of *C. sanguinolenta* by Professor Kwesi Boadu Mensah has been previously described (Mensah-Kane *et al.*, 2020). The cryptolepine used in this study was prepared and stored as previously described (Domfeh *et al.*, 2021). The foetal bovine serum (FBS) (cat # F2442) and Thiazolyl blue tetrazolium bromide powder (MTT powder) (cat # M5655-1G) were purchased from Sigma-Aldrich (St. Louis, USA). Dulbecco's Modified Eagles Medium (DMEM) (high glucose, L-glutamine, sodium pyruvate) (cat # 1-26F58-1) was purchased from BioConcept (Allschwil, Switzerland). Minimum essential medium (MEM) non-essential amino acids (NEAA) (cat # 0823) was purchased from ScienCell (Carlsbad, USA). Opti-MEM reduced serum medium (cat #31985-070) and penicillin-streptomycin (cat #15140) were purchased from Gibco Life Technologies (New York, USA). Isopropyl alcohol (> 99.5% purity, cat # 67-63-0) was purchased from Dae-Jung Chemicals and Metals (Gyeonggi, Korea). The Cignal Finder Reporter Array Plate (cat # CCA-901L) and Attractene Transfection Reagent (cat # 301005) were purchased from Qiagen (Germantown, USA). The Dual-Luciferase Reporter Assay System (cat# E1960) was purchased from Promega (Madison, USA). The manufacturer's instructions were followed in preparing all reagents and chemicals used in this study.

Cell culture

The human embryonic kidney cell line (HEK293, cat # CRL-1573) used in the study was purchased from the American Type Culture Collection (ATCC, Manassas, USA). The cells were grown in DMEM high glucose-containing L-glutamine, sodium pyruvate supplemented with 10% heat-inactivated FBS, 1% NEAA, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37 °C in 5% carbon dioxide (CO_2) under a humidified condition.

Cytotoxicity assay

The HEK293 cells were grown to about 60% confluence and then treated with increasing concentrations of cryptolepine (0 – 10 µM). The cytotoxic effect of cryptolepine was evaluated using an MTT assay at 24 – 72 h post-treatment, as we have previously described (Domfeh *et al.*, 2021).

Cignal Finder 45-Pathway Reporter Array and Reverse Transfection

We used the Cignal Finder 45 Pathway Reporter Array Plate to simultaneously test the ability of cryptolepine to regulate 45 signalling pathways, as we have previously described (Domfeh *et al.*, 2021). These 45 signalling pathways cover research areas such as cancer, immunology, development and toxicology. The Cignal Finder 45-Pathway Reporter Array has 45 pathway reporters dried and coated in duplicate wells of the 96-well plate, with the remaining 6-wells containing positive and negative controls. Each reporter has an inducible transcription factor-responsive construct and a constitutively expressing *Renilla* luciferase reporter gene. The inducible transcription factor-responsive construct

encodes the firefly luciferase reporter gene, monitoring both the increase and decrease in the activity of the coupled transcription factor in said signalling pathway. In contrast, the *Renilla* luciferase reporter gene is an internal control to normalise firefly luciferase activity.

Briefly, the constructs were reverse transfected into HEK293 cells at 8×10^4 cells per well using the Attractene Transfection Reagent. After 24 h of transfection, the cells were treated with cryptolepine (5 µM). At 18 h post-treatment, luciferase assay was performed using the Dual-Luciferase Reporter Assay System. A control experiment in which the cells were reverse-transfected but not treated with cryptolepine was done in parallel with the treated experiment. After preparing the cell lysates, 20 µl of the aliquot was employed for luminescence measurement using a Berthold Orion Luminometer (Berthold Detection Systems, Germany).

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

The HEK293 cells were treated with cryptolepine at increasing concentrations (0 - 5 µM) for 24 h. The total RNA was extracted using the Gene JET RNA Purification Kit (Thermo Scientific, Germany) following the manufacturer's instructions. The total RNA's quantity and purity were verified by spectroscopy (NanoDrop 1000, Thermo Scientific) and 1% agarose gel electrophoresis. As previously described, the total RNA was converted to cDNA (Narkwa *et al.*, 2017; Domfeh *et al.*, 2022). The interferon regulatory factor (IRF1), progesterone receptor (PR), hypoxia-inducible factor-1 alpha (HIF1-α) and signal transducer and activator of transcription 3 (STAT3) transcripts were amplified using the Maxima Probe/Rox qPCR Mix (Thermo Scientific, Germany). Beta-actin (β-actin) was included as an endogenous control. The primers and probes used were designed and synthesised by Biomers, Germany (**Table 1**). The qPCR cycling conditions used were as we previously described (Domfeh *et al.*, 2022). The relative levels of the transcripts (after the normalisation to the transcript levels of β-actin) were derived from the $2^{-\Delta\Delta CT}$ values as done previously (Livak & Schmittgen, 2001).

Statistical Analysis

All the experiments were conducted on three different occasions, and each assay was done in duplicate or triplicate where applicable. The data were analysed using Microsoft Excel (version 2019). Cell viability and differentially regulated pathways were determined as previously described (Domfeh *et al.*, 2021). We used the student's *t*-test to assess differences between treated and untreated cells. In all comparisons, $p < 0.05$ was considered statistically significant.

Table 1: Primers and probe sequences

Target genes	Sequences	Fluorophores
IRF-1	Forward primer:5'-TTTGTATCGGCC TGTGTGAATG-3' Reverse primer:5'-AAGCATGGCTGG GACATCA-3' Probe:5'-CAGCTCCGGAACAAACAG GCATCCTT-3'	5'FAM-3'BHQ1
PR	Forward primer:5'-AGAAATGACTGC ATCGTTGATAAAATC-3' Reverse primer:5'-GGACCATGCCAG CCTGAC-3' Probe:5'-TCTGCCCAGCATGTCGCC TTAGAAAGTGC-3'	5'FAM-3'BHQ1
HIF1-α	Forward primer:5'-CAGAGCAGGAAA AGGAGTCA-3' Reverse primer:5'-AGTAGCTGCATGA TCGTCTG-3' Probe:5'-ACTAGCTTTGCAGAATGCT CAGAGAA-3'	5'FAM-3'BHQ1
STAT3	Forward primer:5'-GGAGCAGAGATG TGGGAATG-3' Reverse primer:5'-GTGGGTCTCTAG GTCAATCTTG-3' Probe:5'-AGTCTCGAAGGTGATCAG GTGCAG-3'	5'FAM-3'BHQ1
β-actin	Forward primer: 5'-TCACCCACACTG TGCCCATCTACGA-3' Reverse primer:5'-CAGCGGAACCGC TCATTGCCAATGG-3' Probe:5'-ATGCCCCCCCCATGCCATC CTGCGT-3'	5'HEX-3'TAMRA

RESULTS

Cytotoxic effect of cryptolepine

We first evaluated the cytotoxic effect of cryptolepine on the HEK293 cells using an MTT assay. It was observed that cryptolepine decreased the viability of the cells in a dose and time-dependent manner (**Fig. 1**).

For the subsequent experiments, cryptolepine concentrations of up to 5 µM were used and with an incubation period of 24 h because the cell viability was more than 80% at these concentrations at 24 h post-incubation (**Fig.1**).

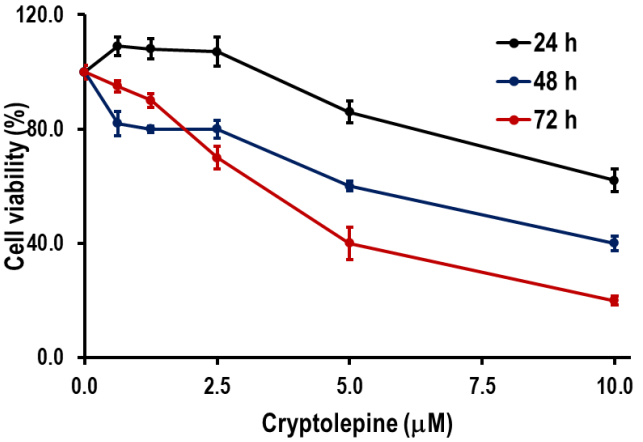

Fig. 1: Toxic effect of cryptolepine on HEK293 cells. The cells were treated with cryptolepine, and the cell viability was assessed at 24 – 72 h using an MTT assay. Data are presented as the means and standard deviations of three independent experiments performed in triplicate wells.

Signalling pathways screened by treating HEK293 cells with cryptolepine

Next, we utilised the Cignal Finder 45-Pathway Reporter Array to screen for cryptolepine effects on 45 signalling pathways in the cells. Out of the 45 signalling pathways, cryptolepine up-regulated nine (9) pathways (**Fig. 2, S1**) and down-regulated twenty-seven (27) pathways (**Fig. 2, S2**). The pathways with fold change values greater than 1.5 and less than 0.7 were considered up-regulated and downregulated, respectively (Protasio *et al.*, 2013; Domfeh *et al.*, 2021).

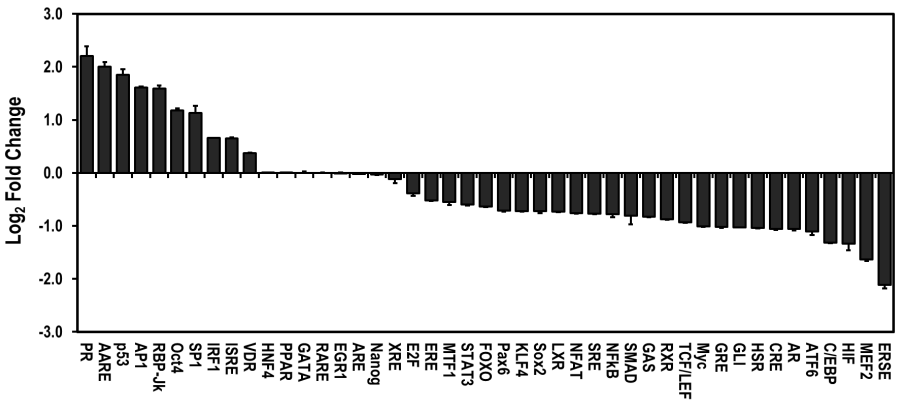

Fig. 2: Cryptolepine-regulated signalling pathways in HEK293 cells. The regulation of the 45 signalling pathways in HEK293 cells after treatment with 5μM of cryptolepine was evaluated using the dual luciferase reporter gene assay. The results were expressed as $\log_2$ of the fold change of the expression of transcription factors between cryptolepine-treated cells and non-treated cells. The error bars represent the standard deviation of three independent experiments conducted in duplicate wells.

Cryptolepine increased the mRNA levels of *IRF-1* and *PR* and decreased the mRNA levels of *HIF-1α* and *STAT3*

Cryptolepine increased *IRF1* and *PR* transcripts in a dose-dependent fashion. At 5µM cryptolepine, the *IRF-1* transcripts were increased by over eight folds (**Fig. 3A**), whereas the *PR* transcripts were increased by over twenty folds (**Fig. 3B**). At 2.5µM and 5µM cryptolepine, the *HIF-1α* transcripts decreased by 70% and 60%, respectively (**Fig. 3C**).

Concerning the *STAT3*, 2.5µM cryptolepine decreased the transcripts by 60%, whereas at 5µM, cryptolepine did not decrease the transcripts (**Fig. 3D**). The up-regulated genes; *IRF1* and *PR*, signal via interferon regulation and progesterone pathways, respectively, whereas the down-regulated genes; *STAT3* and *HIF1-α*, signal via the STAT3 and hypoxia pathways.

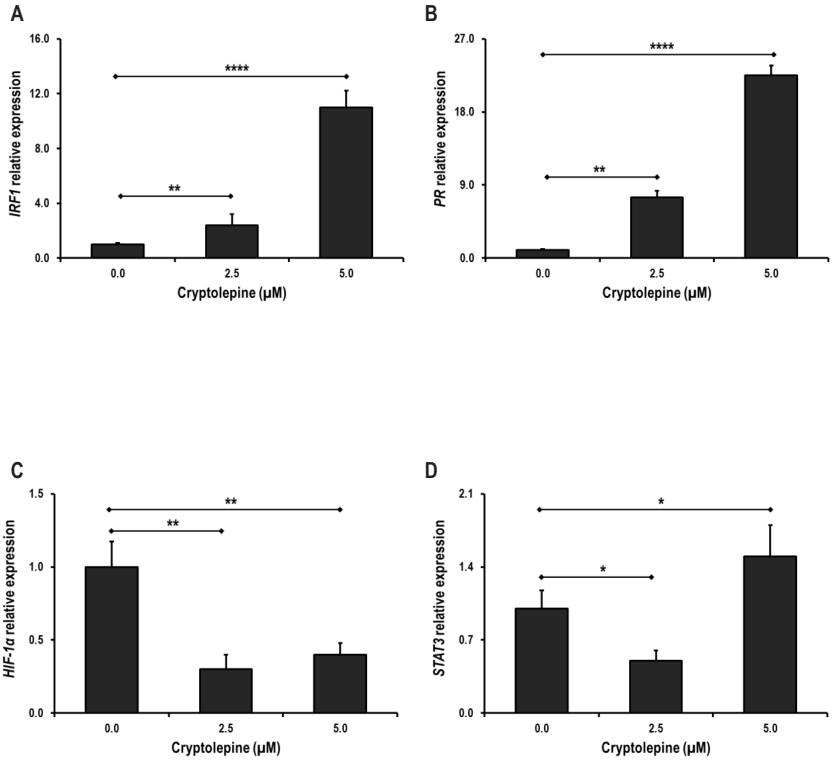

Fig. 3. Effects of cryptolepine on mRNA levels of *IRF1, PR, HIF-1α and STAT3* in HEK293 cells.The cells were treated with cryptolepine, and the relative mRNA levels of the target genes were assessed after 24 h by RT-qPCR with gene-specific primers and probes using β-actin as an endogenous control. Data are shown as the means, and error bars represent the standard deviations. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.0001$ as assessed using the student's *t*-test.

DISCUSSION

We have previously reported that cryptolepine differentially regulates signalling pathways such as p53 and NF-κB in human hepatoma cells (Domfeh *et al.*, 2021). Also, studies have reported that cryptolepine activates several molecules in the p53, NF-κB and apoptotic signalling pathways in different cells (Li *et al.*, 2001; Ansah & Gooderham, 2002; Li *et al.*, 2002; Zhu & Gooderham, 2006). In this current study, cryptolepine up-regulated nine (9) pathways in the HEK293 cells, including progesterone receptor (PR), interferon regulation (IRF1), amino acid deprivation response (AARE), p53/DNA damage and IFN-1 interferon (ISRE) pathways. However, cryptolepine down-regulated twenty-seven (27) pathways, including the STAT3, HIF-1α, NFκB and c-Myc pathways. In our previous study in HepG2 cells (Domfeh *et al.*, 2021), cryptolepine up-regulated seventeen (17) pathways and down-regulated thirteen (13) pathways, which implies that the differential pathway regulation by cryptolepine is cell line dependent even though some pathways were up and down-regulated similarly in both cells.

Next, we focused on the transcript levels of four transcription factors based on their therapeutic potential and our previous studies (Domfeh *et al.*, 2021; Domfeh *et al.*, 2022). Two up-regulated pathway transcription factors were selected for confirmation: interferon-regulated transcription factor (IRF1) and progesterone-regulated transcription factor (PR). Similarly, two down-regulated pathway transcription factors were selected for validation: STAT3-regulated transcription factor (STAT3) and hypoxia-regulated transcription factor (HIF-1α).

IRF1, a protein encoded by *IRF1* in humans, was initially discovered as a transcriptional activator of the IFN system in response to viral infection (Miyamoto *et al.*, 1988; Harada *et al.*, 1989). However, further studies have reported that IRF1 demonstrate great functional diversity and controls the transcription of genes that mediate anti-viral, immunomodulatory, antiproliferative, and anti-cancer effects (Taniguchi *et al.*, 2001; Kröger *et al.*, 2003; Tamura *et al.*, 2008). Hence, the demonstration that cryptolepine induces increased IRF1 transcripts in this current study raises the possibility that IRF1 signalling could be exploited to improve our understanding of IRF1 role in immune response, carcinogenesis and toxicology.

Progesterone receptor (PR) is a transcription factor that mediates the effects of progesterone by associating with a range of co-regulatory proteins and binding to specific target sequences in progesterone-regulated gene promoters (Conneely & Lydon, 2000). Whereas some studies have reported that PR signalling promotes proliferation or differentiation, others have reported that PR signalling inhibits proliferation or differentiation depending on the conditions. Reports indicate that the role of PR signalling in breast tumours may depend on the disease progression stage or the tumour type (Grimm *et al.*, 2016). It has been reported that PR signalling could antagonise the proliferative effects of oestrogen in breast carcinomas (Mohammed *et al.*, 2015). In addition, the presence of PR in primary breast carcinomas has been reported to be a marker of a very positive prognosis and is associated with a less aggressive cancer than PR-negative tumours and the presence of PR in the primary tumour is also associated with better overall survival (Baum *et al.*, 2002; Bardou *et al.*, 2003). We observed in our study that cryptolepine increased the PR transcripts. Given cryptolepine's diverse biological and pharmacological properties in different cells, further investigation is warranted to reveal the effect of cryptolepine on the PR signalling in primary breast carcinomas.

HIF-1α is a major regulatory gene in the cell's response to a reduced oxygen supply

(Semenza, 1999; Semenza, 2000). It plays a significant role in the progression and spread of tumours via the activation of genes linked to the regulation of angiogenesis, cell survival, energy metabolism, and apoptotic and proliferative responses (Wang & Semenza, 1995; Carmeliet *et al.*, 1998; Vaupel, 2004). Studies have shown that tumours that lack HIF-1α significantly reduce vascularisation and growth rates compared to normal cells (Kung *et al.*, 2000; Ryan *et al.*, 2000). Reports indicate that the overexpression of HIF-1α is associated with poor clinical outcomes in patients with various cancers and associated with poor survival in cervical cancer (Birner *et al.*, 2000), endometrial carcinoma (Sivridis *et al.*, 2002), ovarian cancer (Birner *et al.*, 2001), and breast cancer subtypes (Bos *et al.*, 2003; Generali *et al.*, 2006). It has also been reported that HIF-1α has a tissue damage repair response and that it can either turn on or off the critical processes of regeneration of tissues in mammals (Zhang *et al.*, 2015). It has been reported that under-expression of HIF-1α results in healing with a scarring response in mammals (Cho *et al.*, 2015). Hence, the observation in our current study that cryptolepine decreased the HIF-1α transcripts suggests that the effect of cryptolepine on HIF-1α could be exploited in cancer cells and wound healing.

The multitude of evidence in the literature has shown that STAT3 plays a significant role in the development, progression and maintenance of many human tumours (Jing & Tweardy, 2005; Frank, 2007). An increase in activated STAT3 has been shown to correlate with recurrent tumours and poor prognosis of many human cancers (Duan *et al.*, 2006; Rosen *et al.*, 2006). Studies have shown that STAT3-activated genes block apoptosis, promote cell proliferation and survival, enhance angiogenesis and metastasis and inhibit anti-tumour immune response (Jing & Tweardy, 2005; Frank, 2007; Regis *et al.*, 2008). On the other hand, available evidence suggests that any process or approach that disrupts STAT3 signalling may inhibit growth and cause apoptosis in tumour cells and inhibit tumour growth in mouse xenograft cancer models (Jing & Tweardy, 2005; Fletcher *et al.*, 2009; Zhang *et al.*, 2010). Hence, the ability of cryptolepine to reduce the STAT3 transcripts at low concentrations in this study suggests that cryptolepine could be exploited further as an anti-cancer drug agent. This current finding is supported by our previous study, where cryptolepine (1 – 2 μM) inhibited the IL-6/STAT3 signalling pathway in human hepatoma cells (Domfeh *et al.*, 2021).

CONCLUSION

Cryptolepine up-regulated pathways, including the p53, IRF1, ISRE and PR pathways and down-regulated pathways, including the STAT3, c-Myc and HIF-1α pathways. The differential pathway regulations in the HEK293 cells differed from those observed in the HepG2 cells. This study revealed that cryptolepine differentially regulates signalling pathways and regulates these pathways differently in diverse cells. The results from our previous study and this current study support the numerous pharmaco-biological effects of cryptolepine in different cells. Hence, further investigations in varied cells are needed to provide insight into the complex role of cryptolepine in modulating these signalling pathways for therapeutic purposes.

Limitations

The study is limited in that the full effects of cryptolepine on the activated signalling pathways from upstream to downstream were not fully explored due to limited funds. But our previous studies thoroughly explored two pathways (IFN-1 and IL-6/STAT3). Also, the expression of the genes affected by cryptolepine was not confirmed at the protein level.

DATA AVAILABILITY

All the data obtained and analysed are included in this manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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AUTHORS' CONTRIBUTIONS

MM and GA conceived the idea and designed the experiments. PN and SAD performed the experiments, analysed the data and wrote the manuscript. GA provided the funding for the study. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The additional file (**S1 – S3**) contains the reporters, transcription factors, and fold change values of the up-regulated, down-regulated and unaffected signalling pathways.

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