

Original Research Article

Cytotoxic Synergism between a Proprietary *Commiphora mukul* Gum Extract GU-MCT810, 2-deoxy-D-glucose, and Metformin in Human Alveolar Rhabdomyosarcoma and Hepatoma Cell Lines *In vitro*

ABSTRACT

In this investigation we have analyzed the synergism for the cytotoxic effect of a proprietary guggul gum extract (GU), 2-deoxy-D-glucose (2-DG) and metformin (Met) in SJRH30 human alveolar rhabdomyosarcoma and HepG2 hepatoma cell lines *in vitro*. 2-DG and Met as single agents have weak cytotoxic effects in both cell lines. However, the combination of GU+2-DG, GU+Met and 2-DG+Met showed synergism for cytotoxic effect by CompuSyn analysis. Therefore, GU can be included in the combination of drugs involving 2-DG and Met to have synergistic effect. GU also showed a dose-dependent increase in cellular glucose uptake in HepG2 cells like the antidiabetic drug 2,4-thiozolidine dione (TZ). The demonstration of synergism of anticancer effects between GU, metformin and 2-DG, suggest that their mechanisms are in general complementary, though further studies are required to delineate the mechanism of GU, 2-DG and metformin combinations.

Key Words: *Anticancer effect, guggul, 2-deoxy-D-glucose, metformin, rhabdomyosarcoma, hepatoma, cytotoxicity, synergism, glucose uptake*

1. INTRODUCTION

GU-MCT810 (GU) is a proprietary nutraceutical ingredient complex that includes a *Commiphora mukul* (guggul) extract prepared by a supercritical CO₂-co-solvent extraction with ethanol and dissolved in medium-chain triglyceride (MCT) oil composed of C8 and C10 fatty acids. GU was shown to promote hypolipidemic effects *in vitro* as demonstrated by reduction of low-density lipoprotein cholesterol and increased high-density lipoprotein through its direct inhibitory effect on HMG-CoA reductase activity [1]. It was also shown to upregulate the expression of LXR, PPAR, BABP and SHP genes associated with lipid metabolism in the same publication. Additionally, GU inhibits adipocyte differentiation, increases AMPK phosphorylation and AMPK kinase activity and inhibits phosphorylation of mTOR expression [1].

Previously we reported the anticancer effects of GU in combination with hexokinase inhibitor (2-deoxy D-glucose, 2-DG) through the inhibition of HIF-1 α expression in the human HepG2 cell line [2]. Additional anticancer effects were described in a subsequent publication that showed synergism between a proprietary supercritical CO₂ extract of mango ginger (*Curcuma amada* Roxb.) and the hexokinase inhibitor 2-DG and the lactate dehydrogenase-A inhibitor, sodium oxamate, in the U-87MG glioblastoma cell line [3].

Metformin (N,N-dimethylbiguanide) has been an important first-line drug for the treatment of type

2 diabetes (T2D) for decades and generally is regarded as a safe drug [4,5]. It is the most widely used oral antihyperglycemic agent and is currently recommended as first-line therapy for all newly-diagnosed T2D patients [6]. It belongs to the biguanide class of antidiabetic drugs (containing two linked guanidine rings) originally derived from galegine (isoamylene guanidine), a guanidine derivative found in the French lilac, *Galega officinalis*. Besides its glucose-lowering effect, there is interest in its potential relevance to cardiovascular diseases and cancer, although the underlying mechanisms of action remain elusive. Energy metabolism, the target of metformin's mechanism of action in diabetes may also be of importance in cardiovascular diseases and cancer. Accumulating evidence indicates that metformin inhibits growth, survival, and metastasis of different types of tumor cells, including those from breast, liver, bone, pancreas, endometrial, colorectal, kidney, and lung cancers [7].

The anticancer properties of metformin appear to be mediated by AMPK-dependent and -independent pathways. Metformin has been shown to activate AMPK, with compensatory inhibition of mTOR signaling, resulting in suppression of protein synthesis, cell growth and proliferation in neoplastic cells [8]. Suppression of cancer development through AMPK independent activation of autophagy and apoptosis has also been proposed [9]. Other potential mechanisms include suppression of crosstalk between G protein-coupled receptors (GPCRs) and insulin receptor signaling systems that may contribute to the inhibition of pancreatic cancer proliferation [10,11]. Metformin has also been shown to indirectly inhibit cancer proliferation through regulation of angiogenesis, fibroblast and tumor-associated macrophages, and other changes in the tumor microenvironment [12] and through decreased plasma glucose levels that has an inhibitory effect on cancer cell proliferation and survival [13].

2-DG, a synthetic glucose analogue, is a glycolytic inhibitor that is phosphorylated by hexokinase upon transport into the cells and is not fully metabolized [14,15]. 2-DG blocks the first step in glycolysis by inhibiting hexokinase, the first rate-limiting enzyme involved in the conversion of glucose to glucose-6 phosphate. It has been shown to inhibit cell growth in several cancer types and enhance the therapeutic efficiency of chemotherapeutic drugs in human xenograft studies [16-19]. In this paper, we describe the anticancer effect of GU-MCT810, 2-DG and metformin as well as their combinations in alveolar rhabdomyosarcoma and hepatoma cell lines.

2. MATERIALS AND METHODS

2.1 Cell lines and Culture: Human alveolar rhabdomyosarcoma (SJRH30) and hepatoma (HepG2) cell lines were purchased from American Type Culture Collection, Manassas, VA, USA. SJRH30 and HepG2 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 and Dulbecco's Modified Eagle Medium (DMEM), respectively, supplemented with 10% fetal bovine serum (FBS) and antibiotics in a 5% CO₂ incubator.

2.2 Drugs: Metformin (Met), 2-deoxy-D-glucose (2-DG), and 2,4-thiazolidinedione (TZ) and were purchased from Sigma Aldrich Chemical Co., St. Louis, MO. GU-MCT810 was prepared by Flavex Naturextrakte, GmbH, Rehlingen, Germany [1,2] which is formulated to contain 2% guggulsterones in it by dissolving in medium-chain triglyceride oil.

2.3 Cytotoxicity: SJRH30 and HepG2 cells were treated with increasing concentrations of drugs and/or extracts for 72 h in 96 well plates. MTT assay was used to analyze the cytotoxicity of individual drugs/extracts and their combinations [2].

2.4 Glucose uptake: HepG2 cells (4×10^6 cells/4ml) were suspended in DMEM medium on multiwell plates and allowed to grow in the CO₂ incubator. Once the cells were attached, the medium was replaced overnight with a starving DMEM medium containing 0.5% FBS and antibiotics. On the next day, the medium was replaced with fresh starving medium and treated with increasing concentrations of GU or TZ and incubated for 72 h at 37°C. Total cellular protein was extracted with an Invitrogen protein extraction buffer containing protease inhibitors. Cellular extract equivalent to 100 µg protein was analyzed for the glucose content using the Glucose quantitation kit (MBL Laboratories, MA). Cellular glucose concentration was plotted against drug concentrations.

2.5 Data analysis: Mean ICs and standard deviation estimates were calculated using Microsoft Excel software. The fraction of surviving cells at each concentration of drugs/combinations was used for the analysis of synergism/additivity/antagonism between drugs/extracts by the CompuSyn software (ComboSyn Inc, Paramus, NJ). Synergism was evaluated by the combination index (CI) method of Chou and Talalay [20], which is based on the median-effect principle. The CIs at different IC concentrations were calculated by the Chou-Talalay equations for multiple drug effects, which take into consideration both potency (IC values) and shape (slope, m) of the dose-effect curve [21].

3. RESULTS

3.1 Cytotoxicity: Cytotoxicity curves of GU, 2-DG, metformin and different drug combinations in SJRH30 alveolar rhabdomyosarcoma and HepG2 hepatoma cell lines are presented in Fig. 1 and 2, respectively. Also, the inhibitory concentrations of GU, 2-DG, metformin and different drug combinations in SJRH30 alveolar rhabdomyosarcoma and HepG2 hepatoma cell lines are presented in Table 1 and 2, respectively. 2-DG and metformin alone do not demonstrate significant cytotoxicity to SJRH30 and HepG2 cells. However, the combination is synergistic with

respect to cytotoxicity. Similarly, when GU is combined with 2-DG or metformin, cytotoxicity was increased in both SJRH30 and HepG2 cells.

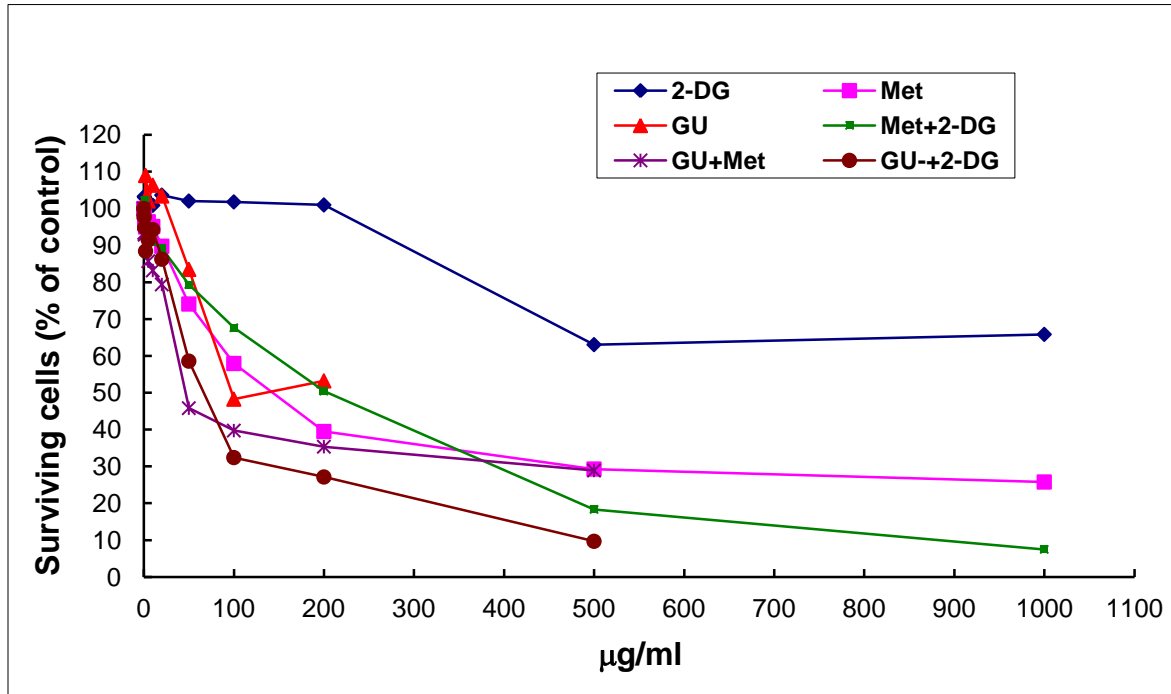


Fig. 1. Cytotoxicity of 2-D-glucose (2-DG), Metformin (Met), GU-MCT810 (GU) and their combinations in SJRH30 rhabdomyosarcoma cell line

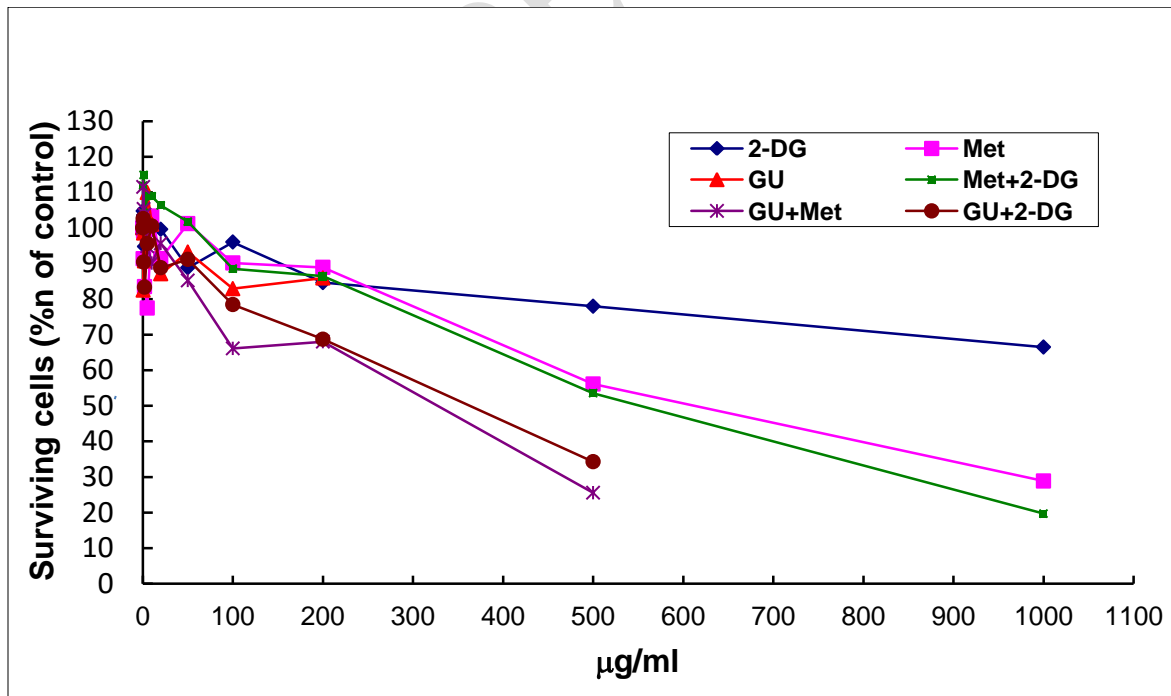


Fig. 2. Cytotoxicity of 2-D-glucose (2-DG), Metformin (Met), GU-MCT810 (GU) and their combinations in HepG2 hepatoma cell line

Table 1. Cytotoxicity of GU-MCT810 (GU), 2-D-glucose (2-DG), metformin (Met) and their combinations in SJRH30 cell line

Drug/combination	IC ₅₀ (µg/ml)	IC ₇₅ (µg/ml)	IC ₉₀ (µg/ml)
GU	50	93	157
2D-Glucose	895	>1000	>1000
Metformin	158	>1000	>1000
GU+2-DG	65	>1000	>1000
GU+Met	46	>500	>500
Met+2-DG	200	470	900

Table 2. Cytotoxicity of GU-MCT810 (GU), 2-D-glucose (2-DG), metformin (Met) and their combinations in HepG2 hepatoma cell line

Drug	IC ₅₀ (µg/ml)	IC ₇₅ (µg/ml)
2-DG	>1000	>1000
Met	605	>1000
GU	>200	>200
Met+2-DG	550	940
GU+2-DG	360	>500
GU+Met	325	498

3.2 CompuSyn Analysis: The median-effect plots of single drugs and drug combinations in SJRH30 cell line is given in Fig. 3A and 3B. Also, the polygonogram indicating the synergism/additivity/antagonism is presented in Fig. 4. The combination index values given in Table 3 indicate that GU+2-DG, GU+Met and Met+2-DG combinations are synergistic for the death of SJRH30 cells. Furthermore, GU+2-DG is more synergistic than GU+Met and Met+2-DG combinations. Essentially all three drugs can be combined against the alveolar rhabdomyosarcoma.

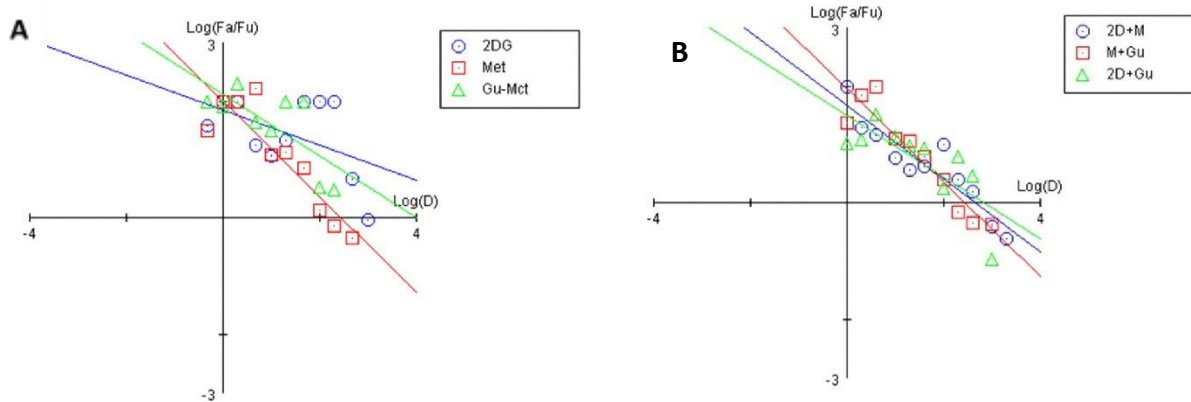


Fig. 3. Median-effect plots of single drug (A) and drug combinations (B) in SJRH30 rhabdomyosarcoma cell line

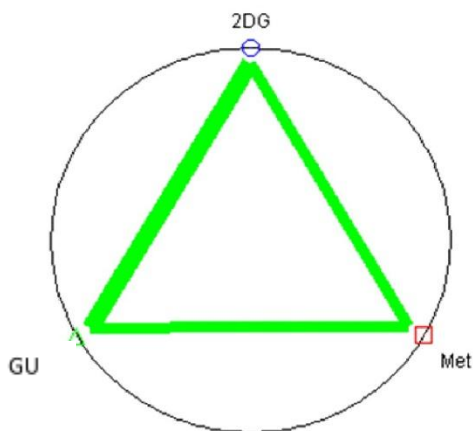


Fig.4. Polygonogram (obtained with CompuSyn analysis) indicating the synergy among GU-MCT810 (GU), 2-deoxy-glucose (2-DG) and metformin (Met) in SJRH30 rhabdomyosarcoma cell line. **The thick green line (GU+2-DG) indicates a higher synergism level than the thin greenline (GU+Met and Met+2-DG).**

Table 3. Combination Index (CI) values for drug combinations calculated with CompuSyn software in SJRH30 rhabdomyosarcoma cell line

Drug combination	CI at IC ₅₀ level	CI at IC ₇₅ level	CI at IC ₉₀ level
GU+2-DG	0.12	0.14	0.17
GU+Met	0.49	0.49	0.51
Met+2-DG	0.79	0.53	0.36

CI <0.1 very strong synergism, 0.1-0.3 strong synergism, 0.3-0.7 synergism, 0.8-0.9 moderate to slight synergism, 0.9-1.1 nearly additive, 1.1-1.45 moderate to slight antagonism, 1.45-3.3 antagonism

Median-effect plots of single drugs and drug combinations in the HepG2 cell line is given in Fig. 5A and 5B. Polygonogram indicating the synergism/addictiveness/antagonism is given in Fig. 6. The combination index values given in Table 4 indicate that GU+2-DG and GU+Met combinations are synergistic for cytotoxicity. The combination index values between Met+2-DG are additive at best at IC_{50} level. Hence, GU must be included in the combination of drugs involving 2-DG and Met in HepG2 cells to have synergistic effect.

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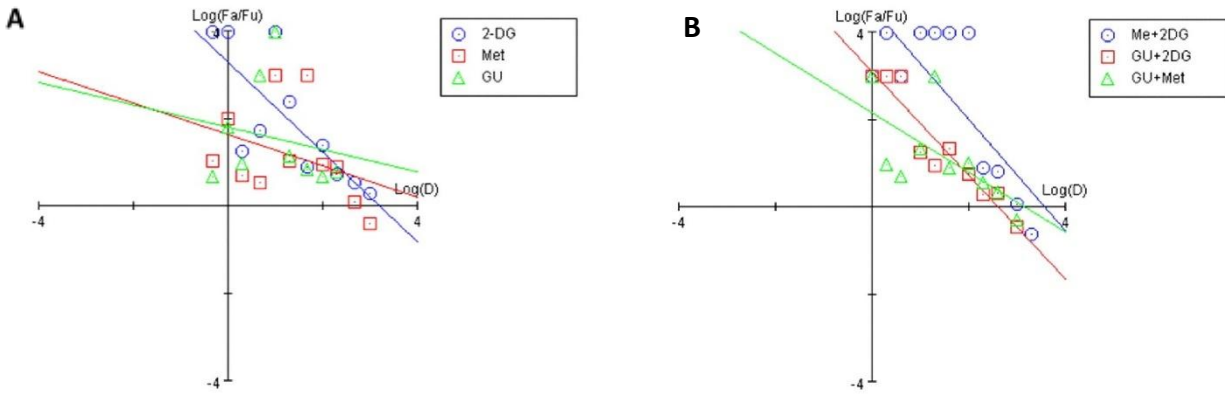


Fig. 5. Medium-effect plot of single drug (A) and drug combinations (B) in HepG2 hepatoma cell line

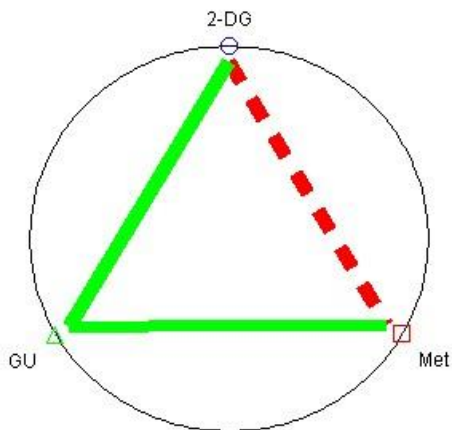


Fig.6. Polygonogram (obtained with CompuSyn analysis) indicating the synergy among GU-MCT810 (GU), 2-deoxy-glucose (2-DG) and metformin (Met) in HepG2 cell line. The green line indicates the synergism and the dotted red line indicates partial additivity. The thick green line (GU+2-DG) indicates a higher synergism level than the thin green line (GU+Met).

Table 4. Combination Index (CI) values for drug combinations calculated with CompuSyn software in HepG2 cell line

Drug	CI at IC ₅₀ level	CI at IC ₇₅ level
Met+2-DG	1.11	1.84
GU+2-DG	0.21	0.22
GU+Met	0.35	0.98

CI <0.1 very strong synergism, 0.1-0.3 strong synergism, 0.3-0.7 synergism, 0.8-0.9 moderate to slight synergism, 0.9-1.1 nearly additive, 1.1-1.45 moderate to slight antagonism, 1.45-3.3 antagonism

3.3 Glucose uptake: Fig. 7 shows the effect of GU and the antidiabetic drug (2,4-thiazolidine dione -TZ) on glucose uptake in HepG2 cells. GU and TZ induced a dose-dependent increase in cellular glucose uptake in HepG2 cells. GU at 50 ug/ml almost doubled the cellular glucose concentration of HepG2 cells.

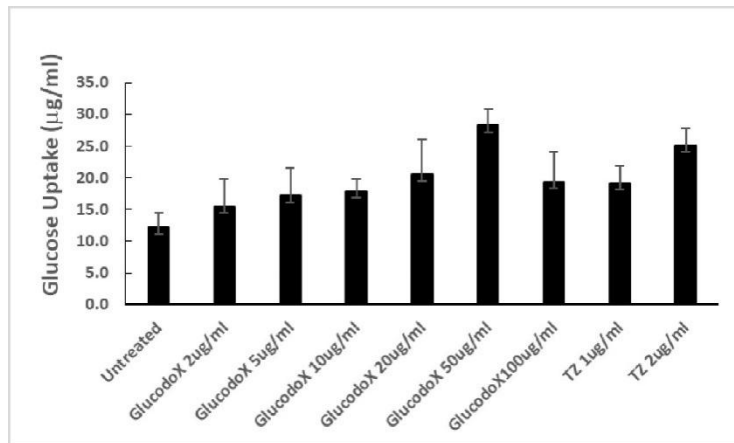


Fig. 7. Effect of GU-MCT810 and 2, 4-thiazide (TZ) on glucose uptake in HepG2 cells.

4. DISCUSSION

GU, a proprietary nutraceutical containing 2% guggulsterones, previously shown to demonstrate hypolipidemic effects through direct inhibition of HMG-CoA reductase activity in a dose-dependent manner [1] has also demonstrated anticancer activity through increased AMPK alpha phosphorylation and AMPK kinase activity and inhibition of mTOR phosphorylation. Given the anticancer activity of metformin [22-24] and 2-DG, via different mechanisms [3,25,26], the object of this study was to determine whether the anticancer effects are synergistic, merely additive or possibly antagonistic. In an earlier publication, we have reported that GU+2-DG combination is useful due to its synergistic antiglycolytic and cytotoxic

effects ([2]. Furthermore, we reported that GU+2-DG combination is useful because of the inhibition of HIF-1 α pathway genes [2]. In the present investigation, GU, 2-DG and metformin have comparatively higher IC values individually in rhabdomyosarcoma and hepatoma cell lines indicating their weak direct cytotoxic effects. However, when the compounds were combined there was a significant reduction in IC values. CompuSyn analysis also showed that these agents work synergistically with respect to cytotoxic effects, indicating a rationale for the combination, compared to single agents. Previously several investigators have shown that 2-DG and metformin can be combined with other front-line cancer drugs for improving chemotherapeutic efficiency [3,18,19,27,28]. The current study suggests that the inclusion of GU could also enhance anticancer efficacy.

Proposing a synergistic combination of these compounds requires several considerations including whether such combinations are safe and practical. 2-DG is a glucose analog that has been shown to act as a competitive inhibitor of glucose metabolism [15]. 2-DG has also been shown to enhance the antitumor activity of Adriamycin and paclitaxel in human xenograft studies [18,25,26]. 2-DG is generally administered intravenously, therefore, in a clinical setting, 2-DG and Metformin or GU could be administered separately as oral preparations. Metformin, on the other hand, is administered orally and has been in use for over half a century and is the most widely prescribed anti-diabetic medication in the world [29]. Therefore, it has an excellent safety profile thus making it appealing for repurposing as an anticancer therapy. Several epidemiologic studies have reported the antitumor effect of metformin in different tumors, such as ovarian [30,31] breast [32] prostate [33] and colorectal [34] cancers. It has been shown to have anticancer effects both in vitro and in vivo [35,36] with the underlying mechanism subject to ongoing investigations. Anticancer properties of metformin result from both direct effects on cancer cells particularly through inhibition of the AMPK/mTOR pathway [22] and indirect effects on the host by virtue of its glucose-lowering properties and anti-inflammatory effects [23,24]. Both mechanisms may be important, although their relative contribution may differ according to cancer stage. We have also shown in the present investigation that the cellular glucose uptake is enhanced with GU treatment in HepG2 cells, which will, in turn, reduce the glucose level in the medium/serum contributing to the anti-diabetic effect. Hence with GU

and metformin treatment it is quite possible to reduce the blood glucose level contributing to the indirect effect of drug combination on the inhibition of cancer cell growth and proliferation.

The concept of repurposing metformin for cancer treatment may be quite appealing [37-39] because it is inexpensive and well-tolerated relative to commonly used antineoplastic agents. However, the antineoplastic activity of metformin requires drug exposure levels considerably higher than those in the serum of metformin-treated diabetic patients. Similarly, 2-DG is also expensive and requires significantly higher doses to have cytotoxic effects on cancer cells [2,3]. Therefore, novel strategies combining these agents with other drugs/extracts exhibiting synergism between them would be preferred over the single-agent use for improved efficacy and reduced toxicity. The demonstration of synergism of anticancer effects between GU, metformin and 2-DG, suggest that their mechanisms are in general complementary, though further studies are required to delineate the mechanism of GU, 2-DG and metformin combinations. Nevertheless, this study has demonstrated the potential that combinations of these compounds are practical and appropriate for investigation in clinical trials.

CONCLUSION

The results of this investigation showed the cytotoxic synergism among supercritical guggul extract (GU), 2-DG and metformin in both SJRH30 alveolar rhabdomyosarcoma and HepG2 hepatoma cells *in vitro*. While GU+2-DG is more synergistic than GU+Met and 2-DG+Met, all three drugs can be combined for the therapeutic management of these cancer types. GU treatment also showed a dose-dependent increase in cellular glucose uptake in HepG2 cells, similar to the antidiabetic drug 2,4-thiozolidine dione.

CONSENT

Not applicable

ETHICAL APPROVAL

Since this investigation does not involve lab animals or human subjects, ethical approval is not applicable.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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