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MINIREVIEWS

Acute left-sided malignant colonic obstruction: Is there a role for endoscopic stenting?

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Abstract

The therapy of left-sided malignant colonic obstruction continues to be one of the largest problems in clinical practice. Numerous studies on colonic stenting for neoplastic colonic obstruction have been reported in the last decades. Thereby the role of self-expandable metal stents (SEMS) in the treatment of malignant colonic obstruction has become better defined. However, numerous prospective and retrospective investigations have highlighted serious concerns about a possible worse outcome after endoscopic colorectal stenting as a bridge to surgery, particularly in case of perforation. This review analyzes the most recent evidence in order to highlight pros and cons of SEMS placement in left-sided malignant colonic obstruction.

Key Words: Colorectal neoplasm; Intestinal obstruction; Endoscopy; Self expandable metallic stents; Colorectal surgery; Chemotherapy

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Core Tip: Self-expandable metal stents (SEMS) should be considered as a primary option in palliative treatment of malignant left-sided colonic obstruction. In patients with conceivably curable left-sided colon cancer, SEMS placement as a bridge to surgery should be carefully discussed, specifically focusing on lower risk and lower permanent stoma rates, but potentially higher recurrence rates when compared to surgery. In this scenario the endoscopic expertise has a significant impact on the complication rate.

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INTRODUCTION

Colorectal cancer (CRC) is the third most frequently diagnosed malignancy in the world and the second cause of cancer-related mortality[1]. CRC is still among the most common reason for large bowel obstruction in adults and about 20% of patients with CRC are admitted with emergency[2-4]. Obstructive CRC most frequently develops in the sigmoid colon, with 75% of tumors located distal to the splenic flexure[5]. Emergency surgery (ES) is the standard approach for obstructive right-sided colon cancer, along with primary resection and ileocolic anastomosis[6]. However, it is debatable whether emergency or radical surgery following stenting as a bridge to surgery (BTS) should be considered for obstructive left-sided colorectal cancer[7]. Self- expandable metal stents (SEMS) for BTS (Figure 1) have shown excellent short-term results, but related complications such as perforations may be disastrous and long-term outcomes are still a matter of debate[8-11].

STENT AS A BRIDGE-TO-SURGERY

Clinical aspects

Over the last decades, many papers have been published on colonic stenting for neoplastic obstruction, including randomized controlled trials (RCT), post-hoc analysis and systematic reviews. Moreover, in 2020 the European Society of Gastrointestinal Endoscopy (ESGE) released updated guidelines on this topic[7]. Even though the role of SEMSs in the management of malignant colonic obstruction has been better defined, several issues still remain. Although screening programs are widespread in developed countries, large bowel obstruction is one of the most common causes of ES in patients with CRC[7,12]. For example, in the United Kingdom, the rate of colorectal cancer presenting as an emergency remains at 20%[13]. Colonic SEMS placement is mainly suggested for patients who have obstructive symptoms and CT-results compatible with obstructing CRC. Acute colorectal obstruction (ACRO) is a medical emergency related to CRC that occurs more frequently in patients with advanced disease, in whom ES is responsible of significant morbidity and mortality than elective surgery, particularly in aged patients[14,15]. These patients usually present to the emergency department with nausea, vomiting, constipation and/or abdominal distention, often combined with poor intake of food from the previous days[16].

In ACRO, the main therapeutic aim is to decrease colonic distension and to prevent complications (i.e. necrosis, perforation), generally associated with pneumoperitoneum and systemic inflammatory response syndrome. Therefore, colonic stenting is an interesting option to obtain this goal in ACRO, as a BTS and for palliative purposes in patients with advanced and/or unfit for surgery CRC[7,15].

Effective stent placement makes it feasible to perform non-surgical intestinal decompression and prepare the colon for a forthcoming elective oncologic resection. Furthermore, in CRC obstruction, the proximal colon is frequently dilated with vascular insufficiency, with an increased risk of colostomy/ileostomy in case of ES. As shown in many studies, in this situation SEMSs may decompress the dilated proximal colon, thus obviating the requirement of ES with colostomy/ileostomy[17].

To evaluate the severity of obstruction, in Japan a modified point score system called ColoRectal Obstruction Scoring System (CROSS) (Table 1) is widely used. CROSS 0 patients need ES or SEMS placement. CROSS 1 or 2 patients are candidates for elective surgery. In CROSS 3 and 4 patients SEMS placement is not required because they can receive food. A post hoc analysis of two prospective, observational, single-arm multicenter clinical trials demonstrated the short-term high efficacy and safety of SEMS placement as a BTS for patients with obstructive CRC classified as CROSS 0, 1, and 2[18].

Clinical success and adverse events

In a large cohort prospective study, the clinical success rate of SEMS placement was 95.5% and the

Table 1 ColoRectal Obstruction Scoring System adapted from Ohki *et al*[18]

Level of oral intake	Score
Requiring continuous decompression	0
No oral intake	1
Liquid or enteral nutrient intake	2
Soft solids, low-residue, and full diet with symptoms of stricure[1]	3
Soft solids, low-residue, and full diet without symptoms of stricure[1]	4

[1]Symptoms of stricture include abdominal pain/cramps, abdominal distension, nausea, vomiting, constipation, and diarrhea, which are related to gastrointestinal transit.

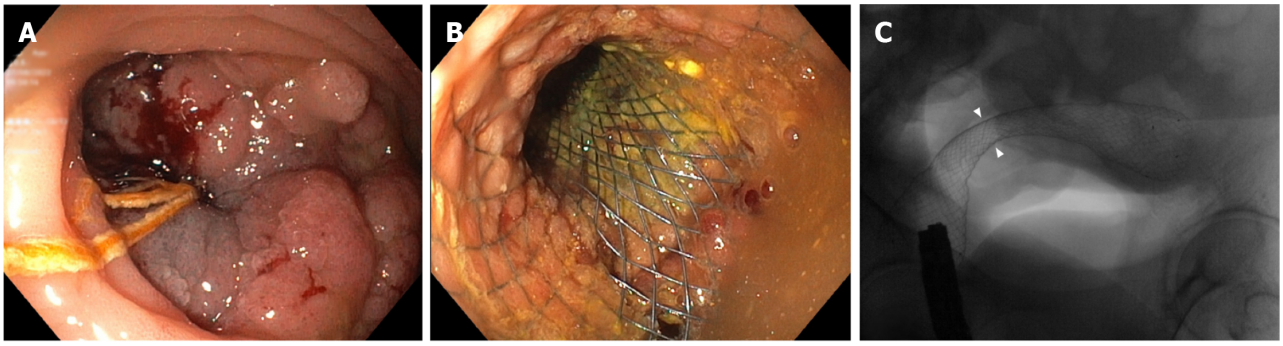

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Figure 1 Left-sided colorectal cancer obstruction treated with self-expandable metal stents. A: Obstructing cancer of the sigmoid colon; B: Endoscopic view after self-expandable metal stents (SEMS) deployment; C: Radiological view of the deployed SEMS.

technical success rate 97.9%. Major adverse events included perforation (2.1%), stent migration (1.0%), and stent occlusion (0.8%)[19]. The primary cause of perforation was the procedure itself (0.8%) followed by comorbidities (impending perforation, obstructive colitis) not manifest prior to SEMS insertion (0.6%). In a retrospective study, the technical success rate for stent placement for left-sided malignant colonic obstruction (LS-MCO) and rectal obstruction did not differ, but the clinical success rate was lower in patients with rectal obstruction (85.4% *vs* 92.1%; $P = 0.02$). In addition, the latter group of patients had a higher complication rate (37.4% *vs* 25.1%; $P = 0.01$), due to an increased risk of extra-intestinal cancer[20]. Furthermore, it is well established from the literature that expertise, method, lesion characteristics, and the location of the obstruction or architecture of the colon, such as tortuosity, have a significant impact on the technical and clinical failure rates for colonic stenting[7,21]. Since there have been growing concerns about protracted and technically challenging stent placement in complex patients, the Colonic Stent Safe Procedure Research Group, in collaboration with the Japan Gastroenterological Endoscopy Society, has developed mini-guidelines to ensure the procedural safety and efficacy for colonic stent placement. A post-hoc analysis[22] of a large multicenter clinical trial identified the risk factors for difficult colonic stenting cases such as a CROSS score of 0 before SEMS placement, evidence of peritoneal carcinomatosis, tumor site in the right colon, stricture length ≥5 cm and placement of multiple SEMSs[22]. In light of this evidence, Kuwai *et al*[22] concluded that before attempting SEMS placement for obstructive CRC clinicians must anticipate technical challenges.

The choice of the stent

Various SEMS have been developed, but they can be classified as covered and uncovered. A recent meta-analysis examined the effectiveness of uncovered vs covered stents in treating colonic obstruction either as a curative BTS or palliative option. Uncovered SEMSs presented less complications (e.g. tumor overgrowth and displacement), longer SEMS patency (mean duration 18 mo), while the risk of tumor ingrowth was higher, as expected. Rates of technical success, clinical success, perforation, stool impaction and stent obstruction were similar in both groups[21].

It is difficult to make recommendations regarding the SEMS length or diameter, as few studies have shown conflicting results. When selecting a stent after fluoroscopic measurement of colonic stricture length, it is widely accepted in clinical practice to follow a simple rule: to prepare for stent foreshortening, the distal edge of the SEMS should be placed proximal to the obstruction. Furthermore, the SEMS length should include 1-2 cm on each side beyond the stricture, considering the extent of shortening once deployed[7,17,21,23].

Is bridge-to-surgery stenting a safe alternative to emergency surgery?

Emergency surgery is burdened by high anastomotic leakage rates, up to 33%[12]. Furthermore a recent study suggests that emergency presentation remains an independent poor prognostic indicator after curative colorectal resection[24]. The optimal management of left-sided malignant large bowel obstruction is less clear than the right-sided cancer where the surgical approach is highly recommended [25].

Several surgical options exist for left-sided bowel obstruction including primary resection (with or without anastomosis), subtotal colectomy (with or without anastomosis) or unfunctioning ileostomy/colostomy with interval resection[24,25].

For the first time in 1994 Tejero *et al*[26] described the technique of SEMS placement in 2 patients with ACRO as a BTS. Nearly twenty years after this initial description, the debate is still open regarding the role of SEMSs as a BTS for symptomatic LS-MCO because interpretation of the literature on this subject is still challenging.

The fundamental hypotheses driving the growing interest in SEMS placement are that it can turn ES into elective surgery, reducing preoperative morbidity. Webster *et al*[25] analyzed 19 international guidelines for the treatment of LS-MCO from 2010 to 2018 and asked whether ES or stent placement as a bridge to surgery was the best procedure in terms of morbidity, mortality and long-term oncological outcomes**.** They concluded that there was a lack of high-quality evidence[25]. The more recent guidelines of the European Society of Gastrointestinal Endoscopy recommend to reserve colonic stenting in case of clinical symptoms and radiological signs of obstructing CRC, without evidence of perforation (strong recommendation, low quality evidence)[7].

In 2011, one of the first multicenter randomized trials comparing ES with colonic stenting as a BTS for left-sided CRC showed that colonic stenting had no decisive clinical advantages for global health status, mortality, morbidity and stoma rates. Moreover their results raised concerns about overt and silent perforations responsible for tumor spread[27].

A systematic review and meta-analysis of RCTs on colonic stenting as a BTS *vs* ES for acute symptomatic malignant left sided colonic obstruction[12] showed that patients treated with SEMS as a BTS had less short-term overall morbidity and reduced rates of both permanent and transient stoma. Albeit influenced by local expertise, level of obstruction and patient's clinical status, stenting as a BTS for LS-MCO showed lower risk than ES in the short-term morbidity (60 d after surgery). However, recurrence rate data between the two groups showed a clear trend in favour of ES over stenting as a BTS (26% *vs* 40%), although this was not statistically significant.

In a subsequent multicenter randomized controlled trial (ESCO trial) comparing stenting as a BTS to ES for malignant colonic obstruction, Arezzo *et al*[28] reported a similar short term complications rate between the two groups but a higher stoma rate in the ES group (P = 0.031). Looking at the long term oncologic results of the ESCO trial, no difference was observed between the two groups in terms of overall survival, time to progression and disease free survival[29]. These results have also been confirmed in a more recent meta-analysis by Cirocchi *et al*[30].

While the majority of studies tried to understand if SEMS placement is more convenient than ES[12,31,32], there are few studies comparing the bridge to elective surgery approach such as decompressive stoma (DS) *vs* SEMS placement. Creation of a DS is a quite simple procedure with a near 100% success rate and can be performed in almost all patients while, as mentioned above, colonic stenting is an intervention requiring specific technical skills and expertise (in both colonoscopy and fluoroscopic techniques), including the ability to select correctly the patient based on stricture's length and location, and carries risks of adverse events. A population-based cohort study[33] comparing the two bridge to elective surgery approaches showed that SEMS appears to be a safest procedure, with a shorter hospital admission, as well as in palliative care. In a recent meta-analysis of seven studies (1 prospective, 6 retrospective), involving 646 and 712 patients who underwent SEMS and DS approaches respectively, Zhang *et al* found a significantly lower complication rate in the SEMS group than in the DS group (8.68 *vs* 16.85%; P = 0.004), without differences in short-term mortality and permanent stoma rates. In line with the previously cited study[33], the authors concluded that SEMSs may be a better alternative to DS for obstructive CRC, but highlighted the lack of high-quality RCTs[34].

Finally, a newly published randomized trial with a longer follow-up (3 y) and larger population compared to prior studies, randomized patients with left-sided obstructive colon cancer to colonic stenting or surgical decompression. The authors showed that among patients undergoing potentially curative treatment, there were no significant differences in 30-d postoperative mortality or duration of hospital stay between stenting followed by delayed elective surgery and emergency surgery group. Moreover the use of a stoma resulted more frequent in patients treated with immediate surgery than in patients treated with SEMS (67.9% *vs* 47.5%; P = 0.003), without substantial differences in peri-operative morbidity, intensive care use, quality of life and 3-y recurrence or mortality[35].

Timing of surgery

The proper timing of surgery subsequent to SEMS placement as a BTS is not clear yet. Adequate radial stent expansion, ischemia reversibility of the colon proximal to the stricture and colon cleansing require sufficient time after SEMS deployment. In order to reduce the risk of stoma and postoperative complications, such as anastomotic leaks, abscesses, and wound's problems, surgery should be postponed for at

least 2 wk after SEMS placement. However, long delays in surgery could increase the complications rate related to SEMS. Therefore, surgery is suggested approximately 14 d after SEMS insertion[7,17].

STENT AS PALLIATIVE TREATMENT

Three randomized controlled trials compared SEMS and decompressive stoma as palliative treatments for malignant bowel obstructions[36-38]. Palliative situations included patients unfit for surgery, as well as patients with inoperable primary lesions or metastatic disease. Given its effectiveness and the enhanced quality of life (QoL) that comes from avoiding a stoma, colonic stenting has been judged to be superior in both investigations. In a randomized prospective trial, Fiori *et al*[37,38] found that the mortality and morbidity rates following palliative stenting and colostomies were comparable. However, in the stenting group a shorter hospital stay, a faster return to oral intake, and a shorter operating time were recorded. On the other hand, a Dutch trial with a similar study design was prematurely stopped because of the unacceptable high mortality rate due to perforations in the stenting group. The authors hypothesized that the unpredictable high frequency of perforation in the nonsurgical arm could be associated with the type of stent used at that time[39].

Stent and chemotherapy

Data about the effects and safety of systemic chemotherapy alone or in association with biological agents (anti-VEGF or anti-EGFR) combined with palliative stenting in metastatic colorectal cancer (mCRC) patients are lacking.

In a metanalysis including 837 mCRC patients, patients treated with SEMS had similar overall survival compared to surgery-treated patients (7.64 mo *vs* 7.88 mo respectively), shorter time before starting chemotherapy (33.36 d *vs* 15.53 d, P < 0.00001) and lower 30-d mortality (4.2% *vs* 10.5%, P = 0.01)[40]. Tumor response to chemotherapy could increase the rate of complications related to stent placement, such as stent migration or late perforation, but, on the other hand, could reduce the risk of obstruction by maintaining its luminal patency, especially in a palliative setting. A multicenter retrospective study included 38 mCRC patients treated with only chemotherapy; major complications related to stenting were: Perforation (8%), stent migration (5%), and re-obstruction secondary to tumor ingrowth (13%)[41]. A retrospective trial including 72 mCRC patients compared long-term outcomes of palliative SEMS in patients treated with chemotherapy or with best supportive care. In the chemotherapy group, there was a higher rate of late migration (20% *vs* 2.4%, P = 0.018, for chemotherapy and best supportive care group respectively); patients refractory to chemotherapy reported a higher rate of late obstruction in comparison to patients who reached disease control during treatment (35.7% in disease progression, 0% in disease control, P = 0.014)[42]. A recent metanalysis evaluated the impact of systemic treatment (chemotherapy alone or in association with targeted therapy) on the risk of complications after SEMS deployment and on outcome in terms of survival rates. Chemotherapy was shown to not be related to a higher risk of SEMS-related complications nor a reduction in the survival rates[43].

The introduction of bevacizumab improved outcome of mCRC patients[44], although data about its effect on stent placement are still controversial. Moreover, some authors raised the hypothesis of an increased risk to develop SEMS-related complications (such as perforation) in patients on bevacizumab [45,46]. Conversely, other authors demonstrated that the addition of bevacizumab to chemotherapy was not related to a higher perforation rate in comparison to chemotherapy alone[47,48]. In an Italian retrospective, multicenter study including 91 mCRC patients treated with chemotherapy plus anti-VEGF or anti-EGFR agents, no correlation between chemotherapy with or without biological therapy, K-RAS status or risk of SEMS-related complications was shown[46].

These studies had several limitations: Retrospective nature, different outcomes and small sample size, patients with heterogeneous characteristics and different settings. At the state of the art more prospective and randomized trials to define the outcome and safety of the association of SEMS placement and systemic treatment are needed.

CONCLUSION

Colonic stenting is a well-recognized palliative approach for treating malignant left-sided colonic obstruction, with high rates of technical and clinical success. Especially in patients with poor general condition and limited life expectancy, it may allow for an early hospital discharge, an improved QoL and prolonged survival in comparison to surgery.

SEMS placement as a BTS has the advantage to convert an ES into an elective one, reducing preoperative morbidity, allowing for adequate oncological staging, good colonic preparation and faster initiation of chemotherapy. Although numerous prospective and retrospective investigations have highlighted serious concerns about tumor seeding after endoscopic colorectal stent placement, partic-

ularly in cases of perforation, recent high quality studies displayed encouraging results. Operator expertise remains a key element to ensure accurate stent placement and restoration of bowel function with a low rate of complications. For this reason, this approach should be considered a standard practice only in experienced high-volume referral centers and clinicians should carefully select the patients fit for an endoscopic decompressing approach before starting the procedure.

In conclusion, further evidence from prospective, ideally randomized trials on the probability of tumor recurrence following stenting is necessary to show the long-term safety of stenting as a BTS. Until then, the evident short-term advantages, combined with the high mortality rate in frail and elderly patients, should be weighed against the potential long-term threats of tumor recurrence.

FOOTNOTES

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MINIREVIEWS

Tyrosine kinase inhibitors and human epidermal growth factor receptor-2 positive breast cancer

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Abstract

The body of evidence investigating human epidermal growth factor receptor-2 (HER2) directed therapy in patients with breast cancer (BC) has been growing within the last decade. Recently, the use of tyrosine kinase inhibitors (TKIs) has been of particular interest in the treatment of human malignancies. This literature commentary is intended to highlight the most recent findings associated with the widely-studied TKI agents and their clinical significance in improving the outcomes of HER2 positive BC.

Key Words: Human epidermal growth factor receptor-2 positive breast cancer; Tyrosine kinase inhibitors; Lapatinib; Pyrotinib; Tucatinib; Trastuzumab

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Core Tip: Newly published randomized controlled trials within the past two years have provided compelling evidence on the use of tyrosine kinase inhibitors (TKIs) such as Lapatinib, Pyrotinib, Neratinib, Tucatinib, Ruxolitinib, and Afatinib. Several of these agents were found to offer better outcomes in terms of progression-free survival when combined with other agents. While some TKIs, namely Lapatinib, and Neratinib, are supported with a large amount of data than others, the medical literature still lacks substantial evidence to draw a clinical conclusion that could modify/add to the present recommendations in human epidermal growth factor receptor-2 positive breast cancer treatment guidelines.

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INTRODUCTION

In 2022, breast cancer (BC) has been the most common cause of cancer-related mortality in women in the United States[1]. Amongst all confirmed BC cases, human epidermal growth factor receptor-2 (HER2) positive BC is estimated to comprise around 15%-20%[2]. Thus, the emergence of HER2-directed therapy, namely, humanized monoclonal antibodies (mAbs), has transformed the path of BC outcomes. The first agent, Trastuzumab, was approved by the United States Food and Drug Administration (FDA) in the past two decades and has revolutionized the treatment modalities[3]. Soon after the approval of other mAbs such as Pertuzumab, and ado-Trastuzumab emtansine, several tyrosine kinase inhibitors (TKIs) have also been approved as targeted therapies[4]. Figure 1 illustrates various TKIs and their targets. Within the last two years (2021 and 2022), significant additions to the literature were made on the use of TKIs in HER2 positive BC. This commentary aims to highlight the most recent findings published in the literature up to this date. Furthermore, since all TKIs, (*e.g.*, Lapatinib, Neratinib, Pyrotinib, and Tucatinib) can be used to treat both early stages and metastatic BC (mBC), either in combination or as monotherapy, their addition to hospital formularies can be of benefit from a pharmacoeconomic perspective[5]. The summary highlighting the ongoing and completed/terminated clinical trials on TKIs in HER2 positive BC patients is given in Table 1.

In a recent phase III randomized controlled trials, dual HER2 blockade with Lapatinib, Trastuzumab, and an aromatase inhibitor (AI) was found to be superior compared to a single HER2 blockade with AI plus Lapatinib alone or Trastuzumab alone in terms of progression-free survival (PFS) in postmenopausal women [hazard ratio: 0.62 (95%CI: 0.45-0.88); $P = 0.0063$][6]. However, this trial was intended to offer an alternative regimen for patients not receiving chemotherapy, a scenario typically followed when chemotherapy is contraindicated[6]. Nevertheless, the question of whether dual blockade with Lapatinib + Trastuzumab combination can be superior to first-line chemotherapy in terms of PFS remained unanswered.

Conversely, in another phase III trial, Pyrotinib + Capecitabine combination was found to yield longer PFS [12.5 mo (95%CI: 9.7–not reached)] as compared to the arm receiving Lapatinib + Capecitabine treatment [6.8 mo (5.4–8.1); hazard ratio 0.39 (95%CI: 0.27–0.56); one-sided $P < 0.0001$][7]. However, unlike the above-mentioned trial, the patient population in this trial was comprised of mBC patients.

Along similar lines, when Neratinib + Capecitabine (N + C) treatment was compared to Lapatinib + Capecitabine (L + C) combination, N + C resulted in longer PFS (Median PFS = 7 mo compared to 5.4 mo; $P = 0.0011$)[8]. Besides, the duration of response (DoR) in N + C *vs* L + C was 11.1 mo *vs* 4.2 mo ($P < 0.0001$), and time to intervention for central nervous system (CNS) illness was 27.9% *vs* 33.8% ($P = 0.039$) in Asian patients with mBC who had previously received at least two HER2-directed regimens[8]. The effectiveness and safety profiles of the N + C combination in the Asian group matched those of the general population. The studies indicated that Neratinib may provide further advantages for HER2+ mBC patients treated with Trastuzumab-only regimens for their metastatic illnesses such as CNS[8].

With the scarcity of published evidence comparing the efficacy of Tucatinib to other TKIs, the question of whether it offers additional PFS benefit was investigated through one network meta-analysis[9]. The data demonstrated that the combination of Tucatinib + Trastuzumab + Capecitabine is regarded as the most effective option in improving both overall survival (OS) and PFS ($P = 0.003$ and $P < 0.0001$). With OS, the choices of Trastuzumab emtansine ($P < 0.004$) and Pertuzumab + Trastuzumab + Capecitabine ($P = 0.011$) are comparatively superior. On the other hand, Neratinib and Lapatinib resulted in greater improvement in PFS ($P = 0.001$) when combined with Capecitabine[9].

However, despite the promising efficacy of Tucatinib over other TKIs, it was associated with increased levels of serum creatinine, which was concerning regarding its effect on renal function. However, the increase in serum creatinine level was found to be attributed to the inhibition of tubular secretion of creatinine[10]. Importantly, one study evaluated the use of Tucatinib *vs* placebo when both were combined with Trastuzumab and Capecitabine. It was concluded that Tucatinib can significantly improve OS (9.1 mo longer in the Tucatinib group) and delay the progression of brain metastasis [hazard ratio, 0.55 (95%CI: 0.36-0.85)][11].

Of note, within the last two years, no additional data regarding Afatinib's use in HER2 positive BC was published. Notably, only one study reported the benefits of Afatinib but the subjects included were not limited to BC, and those included BC patients were not HER2 positive[12]. Thus, there is no significant update regarding Afatinib's role in HER2 positive BC treatment.

Table 1 Main ongoing and completed phase 3 trials evaluating tyrosine kinase inhibitors with HER2+ breast cancer

Study title	Conditions	Interventions	Outcome measures	NCT number
Pyrotinib rechallenge in HER2-positive metastatic breast cancer pretreated with Pyrotinib and Trastuzumab	HER2-positive breast cancer, metastatic breast cancer	Trastuzumab plus chemotherapy: Trastuzumab in combination with Pyrotinib plus chemotherapy	PFS, ORR, AEs	NCT05346861 [14]
A study of Pyrotinib plus Capecitabine in patients with HER2+ metastatic breast cancer	HER2 positive metastatic breast cancer	Pyrotinib, Capecitabine	PFS, ORR, AEs, SAEs, DoR, CBR, OS	NCT02973737 [15]
A randomized controlled trial of HER2 positive breast cancer patients treated with Lapatinib *vs* herceptin	HER2-positive breast cancer	Lapatinib/Herceptin	DFS, OS	NCT03085368 [16]
Tykerb evaluation after chemotherapy (TEACH): Lapatinib versus placebo in women with early-stage breast cancer	Neoplasms, breast	Lapatinib	This clinical trial has several outcomes measures to be evaluated including DFS, OS, MDFS	NCT00374322 [17]
Neo altto (neoadjuvant Lapatinib and/or Trastuzumab treatment optimization) study	Neoplasms, breast	Lapatinib, Trastuzumab, Paclitaxel	This clinical trial has several outcomes measures to be evaluated including OS, Par with pCR at the ToS, OR at the ToS	NCT00553358 [18]
Lapatinib in combination with Trastuzumab versus Lapatinib monotherapy in subjects with HER2-positive metastatic breast cancer	Neoplasms, breast	Lapatinib, Trastuzumab	PFS, OS, OR, CBR, TTR, DR, change from baseline in FACT-B scores at week 4, week 12, week 16, week 24, and conclusion or withdrawal from study	NCT00320385 [19]
Paclitaxel with/without GW572016 (Lapatinib) as first line therapy for women with advanced or metastatic breast cancer	Neoplasms, breast	Paclitaxel, GW572016 (Lapatinib)	This clinical trial has several outcomes measures to be evaluated including PFS, OS, DoR	NCT00075270 [20]
Continued HER2 suppression with Lapatinib plus Trastuzumab *vs* Trastuzumab alone (terminated)	Cancer	Lapatinib, Trastuzumab	PFS, OS, Best overall response, CBR (CR, PR or SD ≥ 24 wk), AE	NCT00968968 [21]

PFS: Progression-free survival; ORR: overall response rate; AEs: Adverse events; SAE; serious adverse events; DoR: Duration of response; OS: Overall survival; CBR: Clinical benefit rate; MDFS; Modified disease-free survival; Par: Number of participants; TTR: Time in the therapeutic range; DR: Duration of response; pCR: Pathological complete response; DFS: Disease-free Survival; FACT-B: Functional assessment of cancer therapy-breast cancer; OR: Overall response; ToS: Time of surgery; NCT: National clinical trial.

With Ruxolitinib, a class of the Janus kinase inhibitors, the first and only study performed so far with a Trastuzumab combination indicated that the tolerability data is appealing[12]. However, there was no difference in the PFS than that of Trastuzumab alone in mBC patients as compared to the historical control[13]. To draw a more robust conclusion regarding Ruxolitinib and explore its implications with TKIs, more interventional studies are warranted with larger power using randomized and prospective designs since these aspects are lacking in Ruxolitinib studies.

CONCLUSION

In conclusion, while the body of evidence currently available in the literature is still insufficient to offer recommendations in the treatment guidelines of HER2 positive BC, the existing studies concluding the benefits of TKIs promise hope for patients resistant to conventional first- and second-line treatments.

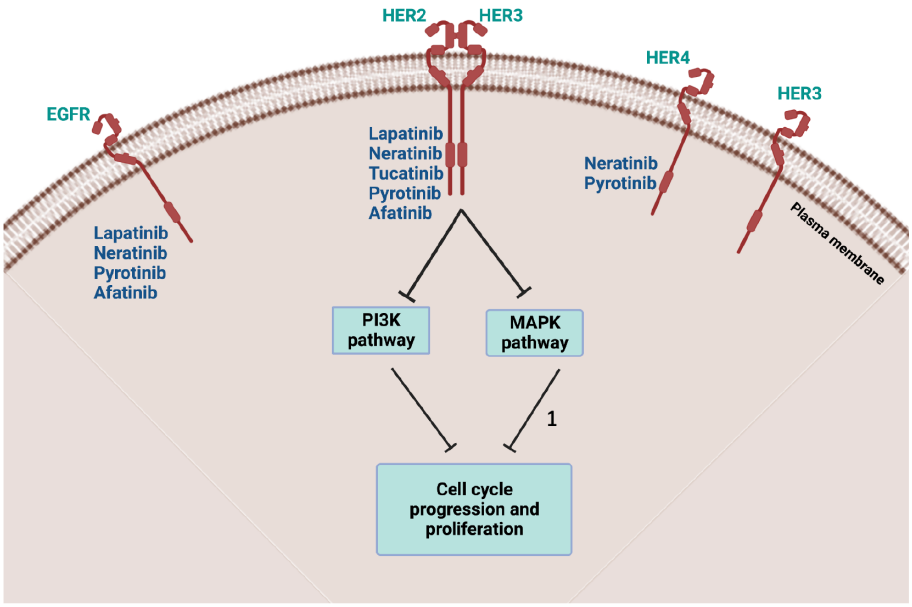

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Figure 1 Schematic representation of TKIs targeting EGFR and various HER family receptors, leading to the inhibition of downstream PI3K and MAPK pathway, resulting in the regulation of cell cycle progression and proliferation. [1]The sign denotes inhibition. The authors would like to acknowledge Biorender.com software that was used to create Figure 1.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Thymoquinone enhances the antioxidant and anticancer activity of Lebanese propolis

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Abstract

BACKGROUND

Reactive oxygen species (ROS) are produced by multiple cellular processes and are maintained at optimal levels in normal cells by endogenous antioxidants. In recent years, the search for potential exogenous antioxidants from dietary sources has gained considerable attention to eliminate excess ROS that is associated with oxidative stress related diseases including cancer. Propolis, a resinous honeybee product, has been shown to have protective effects against oxidative stress and anticancer effects against several types of neoplasms.

AIM

To investigate the antioxidant and anticancer potential of Lebanese propolis when applied alone or in combination with the promising anticancer compound Thymoquinone (TQ) the main constituent of *Nigella sativa* essential oil.

METHODS

Crude extracts of Lebanese propolis collected from two locations, Rashaya and Akkar-Danniyeh, were prepared in methanol and the total phenolic content was determined by Folin–Ciocalteu method. The antioxidant activity was assessed by the ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and to inhibit H_2O_2-induced oxidative hemolysis of human erythrocytes. The anticancer activity was evaluated by [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] MTT assay against HCT-116 human colorectal cancer cells and MDA-MB-231 human breast cancer cells.

RESULTS

The total phenolic content of propolis extract from Rashaya and Akkar-Danniyeh were 56.81 μg and 83.503 μg of gallic acid equivalent /mg of propolis, respectively. Both natural agents exhibited strong antioxidant activities as evidenced by their ability to scavenge DPPH free radical and to protect erythrocytes against H_2O_2-induced hemolysis. They also dose-dependently decreased the viability of both cancer cell lines. The IC_{50} value of each of propolis extract from Rashaya and Akkar-Danniyeh or TQ was 22.3, 61.7, 40.44 μg/mL for breast cancer cells at 72 h and 33.3, 50.9, 33.5 μg/mL for colorectal cancer cells at the same time point, respectively. Importantly, the inhibitory effects of propolis on DPPH radicals and cancer cell viability were achieved at half its concentration when combined with TQ.

CONCLUSION

Our results indicate that Lebanese propolis extract has antioxidant and anticancer potential and its combination with TQ could possibly prevent ROS- mediated diseases.

Key Words: Lebanese propolis; Thymoquinone; Combination; Antioxidant activity; Anticancer activity; Phenolic compounds

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Core Tip: Combining Thymoquinone with Lebanese propolis enhanced its antioxidant activity and its anticancer effects against breast and colorectal cancer cells. The combination of these natural products could have potential health benefits and could possibly prevent oxidative stress mediated diseases including cancer.

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INTRODUCTION

Oxidative stress refers to the imbalance between the generation of reactive oxygen species (ROS) and their neutralization by endogenous antioxidant systems resulting in an excess of ROS which has detrimental effects on key cellular components[1,2]. There are two types of ROS: Free radicals and nonradicals. Free radicals are highly reactive molecules because they have at least one unpaired electron in their structure and react with different biological macromolecules[3]. Although nonradical species are less reactive than free radicals, they can easily cause free radical reactions in living organisms[3,4]. The accumulation of ROS causes the peroxidation of cell membrane lipids and cell membrane disintegration, alters the configuration of proteins resulting in loss of biochemical functionality in addition to inducing DNA mutations and replication errors[2]. Ample evidence shows that ROS-mediated oxidative stress is associated with the pathogenesis of various diseases including cancer, cardiovascular diseases, neurodegenerative disorders, and diabetes[5].

Removing excessive ROS by exogenous antioxidants supplementation has long been considered a potential strategy to prevent diseases. Over the last decade, there has been considerable interest in the intake of natural antioxidants from food and diets to strengthen cell antioxidant defense in humans. A recent pilot study demonstrated that a healthy mixed diet rich in antioxidant micronutrients reduced the concentration of ROS in the blood of healthy subjects[6]. Another study showed that regular consumption of an antioxidant-rich juice increased plasma antioxidant capacity and reduced plasma lipid oxidation in healthy individuals[7]. In addition, several clinical trials showed that intake of foods rich in antioxidants can potentiate plasma antioxidant capacity and reduce oxidative stress markers in subjects with diabetes, obesity, and dyslipidemia[8]. Interestingly, the combination of several antioxidants has been suggested to be more potent than the application of single antioxidants given the diverse chemistry and biochemistry of ROS, and the interactions that could arise from antioxidants that have different modes of action[9].

Propolis is a glue-like resinous material produced by honeybees from various plant sources and used in the construction and maintenance of their hives[10,11]. Propolis possesses numerous health-promoting potentials including anti-inflammatory[12], antioxidant[13], anticancer[14] and antidiabetic effects[15]. The chemical composition and therefore the biological effects of propolis vary depending on

several factors such as the geographical region, botanical source, and the bee species[16]. The bioactive compounds of propolis were reported to effectively scavenge free radicals[17]. Different *in vivo* studies reported the protective effects of propolis against the oxidative stress induced by several exogenous oxidants such as cisplatin[18], isoproterenol[19], nicotine[20], UV[21], and carbon tetrachloride[22]. In addition, propolis was demonstrated to reduce the blood pressure and suppress oxidative stress in heart, liver, and renal tissues in animal models of hypertension[23-25].

Thymoquinone (TQ), the major bioactive constituent of *Nigella sativa* (black seed) essential oil, was extensively studied for its diverse therapeutic benefits including antioxidant, anti-inflammatory, anticancer, antibacterial, antifungal and anticonvulsant activity[26]. TQ was reported as a strong scavenger of different ROS and was found to inhibit non-enzymatic lipid peroxidation[27]. TQ was demonstrated to have a protective effect against oxidative stress induced in rats by different agents such as radiation[28], lead[29] and acrylamide[30]. In addition, it reduced the oxidative stress in rat models of myocardial infarction[31], diabetes mellitus[32], lung injury[33] and dopaminergic neurodegeneration [34].

Although the antioxidant potential of propolis and TQ has been well investigated in previous studies, there are no studies that have evaluated the antioxidant and anticancer effects of the combination of both natural agents. Thus, we aimed to test the antioxidant and anticancer activities of combining TQ and propolis that was collected from two locations in Lebanon (Rashaya and Akkar-Danniyeh). We evaluated the total phenolic content of both propolis extract and determined the antihemolytic and antioxidant activity of propolis and TQ in addition to their anticancer effects against HCT-116 human colorectal cancer cells and MDA-MB-231 human breast cancer cells.

MATERIALS AND METHODS

Preparation of thymoquinone

Fresh stocks of the purified synthetic compound TQ (Sigma-Aldrich) were prepared in methanol directly before use.

Preparation of methanol extracts of propolis

Two samples of raw propolis material were collected, the first from Rashaya district in the Beqaa governorate of Lebanon and the second from Akkar-Danniyeh in the north of the country. A mass of 10 g of raw propolis from each sample was chopped into small pieces and extracted with 100 mL distilled water. The extraction was carried at 80°C for 3 h and the obtained solution was subsequently filtered through a Buchner funnel. Residues were then extracted with 100 mL methanol. The extraction was carried at room temperature for 4 h then at 50°C for 15 min. The propolis extracts were subsequently filtered three times by Buchner funnel. The obtained filtrate was evaporated by nitrogen gas to obtain the methanol propolis extract (MPE). MPE-R denotes MPE from Rashaya and MPE-D denotes MPE from Akkar-Danniyeh.

Total phenolic content

The relative content in phenols was determined according to the Folin Ciocalteu method. Briefly, 100 µL of MPE-R or MPE-D (1 mg/mL of methanol) from each sample were mixed with 500 µL of Folin Ciocalteu's phenol reagent 10%. After 5 min, 1.5 mL of 2% sodium bicarbonate were added to the solution. The mixture was maintained at room temperature in the dark for 30 min after which the absorbance was recorded at 760 nm using a spectrophotometer. The total phenolic content was calculated using the calibration curve generated from standard solutions of gallic acid ranging from 0 to 50 µg/mL ($y = 0.2811x - 0.3266$; $R^2 = 0.956$). Total phenolic content was expressed as the average of 3 independent experiments performed in triplicates and as µg of gallic acid equivalents (GAE)/mg of propolis.

DPPH assay

Free radical-scavenger activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Briefly, 1 mL of MPE-R or MPE-D (10-100 µg/mL) were mixed with 1 mL of DPPH (0.052 mg/mL methanol). The reaction mixtures were homogenized and incubated in the dark at room temperature for 30 min and the absorbance (Abs) was measured at 515 nm by a Gene Quant 1300 UV-Vis spectrophotometer. The ascorbic acid was used as a reference antioxidant and a mixture of 1 mL DPPH with 1 mL methanol was used as a control. For combination treatments, TQ (12.5-100 µg/mL) was combined with MPE-R or MPE-D (10-50 µg/mL) and the experiment was carried as described above. The DPPH scavenging ability of the different agents was calculated using the following equation: % DPPH inhibition = [(Abs control − Abs sample)]/ (Abs control)] × 100.

H_2O_2- induced hemolysis

Fresh human blood was washed three times with 1X phosphate-buffered saline (PBS). With every wash,

the sample was centrifuged for 12 min at 4°C and 2500 rpm, the supernatant was discarded, and the pellet was resuspended in PBS. Then, the pellet was resuspended in Dulbecco's PBS and 1 mL of the cell suspension was mixed with 100 µL of each of MPE-R, MPE-D or TQ at 10, 50, and 100 µg/mL. After 5 min, 1 mL of 10% H_2O_2 was added, and the mixture was incubated at 37°C for 90 min and shaken every 30 min. This was followed by centrifugation at 4°C and 2500 rpm for 10 min and measurement of the absorbance of the supernatant at 540 nm. The positive control consisted of a mixture of blood with 10% H_2O_2. The results were expressed as percentage of inhibition of hemolysis. % inhibition of hemolysis= [(Abs control − Abs sample)]/(Abs control)] × 100

Hemolytic activity

Fresh human blood was washed three times with 1X PBS. With every wash, the sample was centrifuged at 4°C and 2500 rpm for 12 min, the supernatant was discarded, and the pellet was resuspended in PBS. The washed blood was mixed with each of MPE-R or MPE-D (10, 100, 200 µg/mL), TQ (20, 50, 100 µg/mL) or their combinations. The mixture was kept at 37°C for 90 min and was shaken every 30 min. The samples were then centrifuged at 4°C, 2500 rpm, for 10 min after which the absorbance of the supernatant was recorded at 540 nm. The positive control consisted of a mixture of blood with 1% SDS which is known to cause hemolysis. The results were expressed as the percentage of hemolysis. % hemolysis= [Abs sample/ Abs control] × 100

Cell culture conditions

HCT-116 human colorectal cancer cells and MDA-MB-231 human breast cancer cells were maintained in RPMI 1640 (Lonza; Cat.N: BE12-115F) supplemented with 10% fetal bovine serum (Sigma F9665) and 1% penicillin/ streptomycin (Sigma, P4333) in a humidified atmosphere at 37°C in 5% CO_2.

MTT cell viability assay

HCT-116 and MDA-MB-231 cells were seeded overnight in 96-well microtiter plates at a density of 10^4 cells/well. After 24 h, cells were treated with MPE-R, MPE-D or TQ at a concentration ranging from 1-15 µg/mL or with the combination of MPE-R or MPE-D (0.5-7.5 µg/mL) with TQ (0.5-7.5 µg/mL). After 24, 48 and 72 h of treatment, cells in each well were incubated with 20 µL of [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] MTT for 3 to 4 h, then with 100 µL of DMSO for about 1 h. The MTT optical density (O.D.) was then measured by a microplate spectrophotometer at 515 nm. The results are expressed as percentage of viable cells with respect to the untreated control using this formula: % viability = [mean O.D. treatment/mean O.D. control] × 100.

Statistical analysis

Data are presented as means ± SD. Two tailed Student's *t*-test was performed to evaluate the statistical significance of the difference between the groups using GraphPad Prism V.9.5.0 software. Statistical significance was set with a 95% confidence interval at $P < 0.05$, $P < 0.01$ and $P < 0.0001$.

RESULTS

Total phenolic content of propolis varies depending on location

The total phenolic content of propolis extracts was determined by Folin Ciocalteu method and is reported as gallic acid equivalents by reference to a standard curve (y = 0.2811x - 0.3266; $R^2 = 0.956$). The phenolic content was variable depending on location such that the total phenolic content of MPE- D in 1 mg of propolis was 47% higher than that of MPE-R(Table 1).

TQ enhanced the antioxidant activity of propolis

We then evaluated the ability of propolis extracts to scavenge free radicals using DPPH radical scavenging assay. Both propolis extract exhibited a dose-dependent DPPH inhibition efficiency suggesting antioxidant potential. MPE-D had higher antioxidant activity than MPE-R as reflected by the higher percentages of inhibition recorded at all the concentrations ranging from 20-100 µg/mL. MPE-R showed maximum inhibition of DPPH of 56.5% at 100 µg/mL, while inhibition by MPE-D reached 89% at 75 and 100 µg/mL (Figure 1A).

To determine whether the antioxidant effects of propolis extracts could be potentiated by TQ, we combined each of MPE-R or MPE-D (10-50 µg/mL) with TQ (12.5-100 µg/mL) and evaluated their antioxidant activity in comparison to single treatments. Results showed that the combination with TQ enhanced the antioxidant activity of propolis extracts. While a dose of 100 µg/mL of MPE-R induced 56.5% inhibition of DPPH, the combination of 50 µg/mL of MPE-R with 100 µg/mL TQ caused 85.7% inhibition. MPE-D alone showed maximal inhibitory effects of 89% at 75-100 µg/mL, while combination with 50-100 µg/mL TQ resulted in 84% inhibition at lower concentrations of 25-50 µg/mL (Figure 1B).

Table 1 Total phenolic content of methanol propolis extract from Rashaya and Akkar-Danniyeh in µg of gallic acid equivalents/mg of propolis and µg/mL of methanol propolis extract

	TPC (µg GAE/mg)	TPC (µg GAE/mL of MPE)
MPE-R	56.81	2.3
MPE-D	83.503	3.997

TPC: Total phenolic content; MPE: Methanol propolis extract; MPE-R: Methanol propolis extract from Rashaya; MPE-D: Methanol propolis extract from Akkar-Danniyeh; GAE: Gallic acid equivalents.

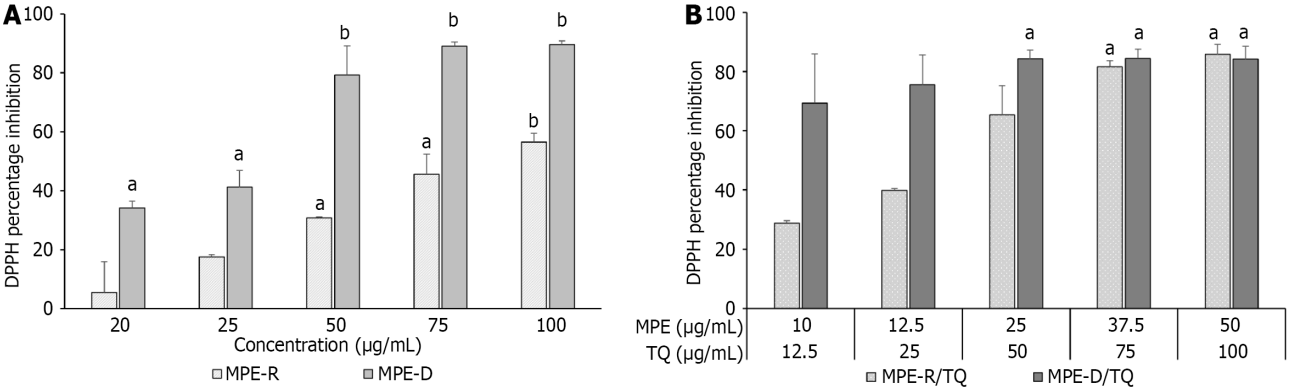

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Figure 1 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity of methanol propolis extract alone or in combination with Thymoquinone. A: 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity of each of methanol propolis extract from Rashaya (MPE-R) and from Akkar-Danniyeh (MPE-D; 20-100 µg/mL) alone; B: DPPH free radical scavenging activity of each of MPE-R and MPE-D (10-50 µg/mL) in combination with Thymoquinone (TQ; 12.5-100 µg/mL). The samples were mixed with DPPH and the absorbance of the mixture was measured after 30 min. The values are expressed as percentage of DPPH percentage inhibition relative to the control. Each value represents the mean ± SD of $n = 2$ experiments. $^{a}P < 0.05$ and $^{b}P < 0.01$ are significantly different from control using two-tailed Student's *t*-test.

Propolis extracts and TQ protected human red blood cells against oxidative hemolysis

We then evaluated the biological relevance of the antioxidant activity of propolis extracts and of TQ by testing the protective effects of single treatments against oxidative hemolysis induced by H_2O_2 in human red blood cells. Treatment with MPE-R, MPE-D or TQ exhibited good antihemolytic potential against H_2O_2-induced hemolysis. A dose of 10 µg/mL of each of MPE-R, MPE-D and TQ induced 46, 49 and 51% decrease in hemolysis, respectively (Figure 2).

The combination of propolis extracts with TQ had no hemolytic activity at low concentrations

To investigate if propolis extracts or TQ are toxic to human red blood cells, we evaluated their hemolytic potential at concentrations ranging from 10- 200 µg/mL and 20- 100 µg/mL, respectively. Both MPE-R and MPE-D produced less than 5% hemolysis at 10 µg/mL, suggesting that these extracts are not toxic to red blood cells at this concentration. Increasing concentrations of MPE-D up to 100 or 200 µg/mL also showed low hemolytic activity of 7.8%. Similarly, hemolysis by TQ was less than 5% at all the tested concentrations. However, MPE-R induced higher hemolytic response that reached 20% at 200 µg/mL (Figure 3A).

Combining 5 µg/mL MPE-R or MPE-D with 10 µg/mL TQ produced less than 5% hemolysis suggesting that combinations at these low doses have low hemolytic effects. However, increasing concentrations to 50 µg/mL MPE-R and 25 µg/mL TQ or 100 µg/mL MPE-R and 50 µg/mL TQ produced 12.7% and 21.9% hemolysis, respectively. Similar concentrations of MPE-D and TQ produced 7.3% and 13.7% hemolysis, respectively (Figure 3B), suggesting that MPE-R had higher hemolytic effects when combined with TQ at higher doses.

TQ potentiated the inhibitory effects of propolis extracts on cancer cell viability

Next, we tested the anticancer activity of propolis extracts when applied alone or in combination with TQ. MDA-MB-231 human breast cancer cells and HCT-116 human colorectal cancer cells were treated with different concentrations of the natural products for 24, 48 and 72 h after which cell viability was assessed by MTT assay. Single treatments with MPE-R or MPE-D (1-15 µg/mL) reduced the viability of both cell lines in a dose dependent manner to almost similar levels. Treatment of MDA-MB 231 cells with 15 µg/mL of MPE-R, MPE-D or TQ for 72 h caused 34.6%, 18.5% and 24.52% reduction in cell

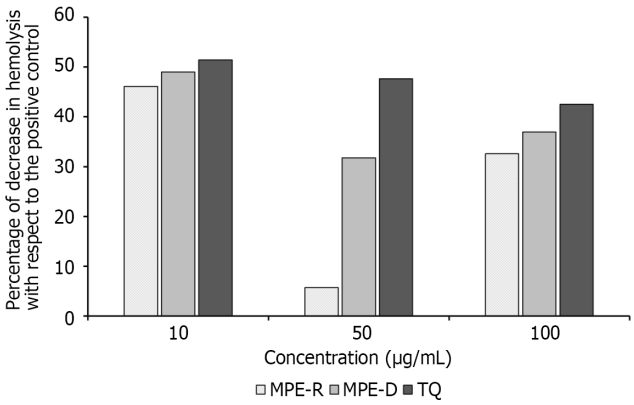

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Figure 2 *In vitro* antihemolytic/cytoprotective activity of each of Thymoquinone and methanol propolis extract from Rashaya and Akkar-Danniyeh against H_2O_2- induced oxidative hemolysis. Human red blood cells suspension was preincubated with methanol propolis extract from Rashaya (MPE-R), methanol propolis extract from Akkar-Danniyeh (MPE-D), or Thymoquinone (TQ; 10-100 µg/mL) for 5 min. The cell suspension was then incubated with 10% H_2O_2 for 90 min at 37°C. The samples were then centrifuged, and the absorbance of the supernatant was measured. The values are expressed as percentage of decrease in hemolysis with respect to the positive control (10 % H_2O_2). Each value is obtained from n = 1 experiment performed in monoplicate.

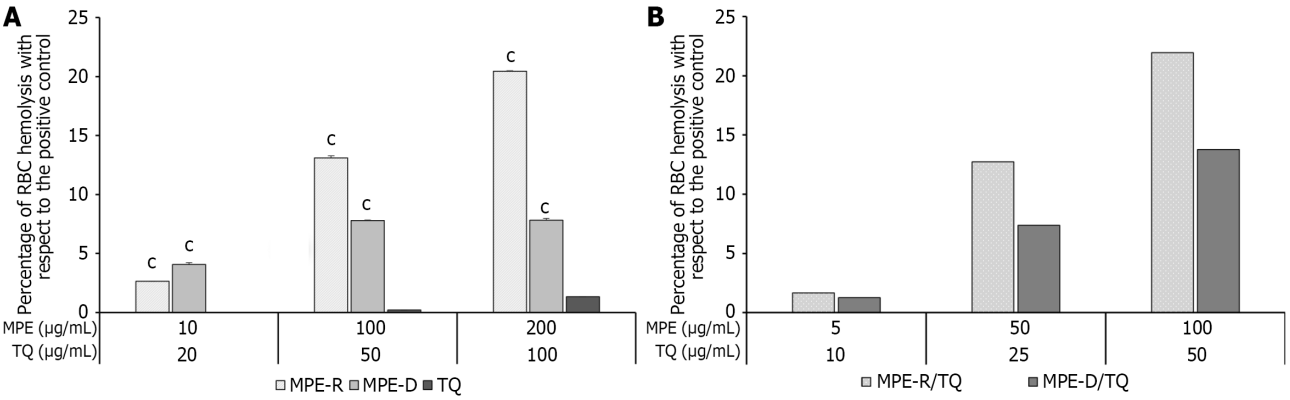

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Figure 3 *In vitro* hemolytic activity of each of methanol propolis extract from Rashaya and Akkar-Danniyeh alone or in combination with Thymoquinone. A: Hemolytic activity of each of methanol propolis extract from Rashaya (MPE-R) and methanol propolis extract from Akkar-Danniyeh (MPE-D; 10-200 µg/mL) and Thymoquinone (TQ; 20-100 µg/mL); B: hemolytic activity of the combination of MPE- R or- D (5-100 µg/mL) and TQ (10-50 µg/mL). Washed fresh human blood was incubated with the natural products for 90 min. The samples were then centrifuged, and the absorbance of the supernatant was measured. The values are expressed as percentages of red blood cells hemolysis with respect to the positive control (SDS 1%). Each value represents the mean ± SD of n = 3 experiments for MPE-R and MPE-D single treatments and n = 1 for TQ single treatment and combination treatments. $^cP < 0.0001$ is significantly different from positive control using two-tailed Student's t-test.

viability, respectively. The IC_{50} value of each of MPE-R, MPE-D or TQ at 72 h was 22.3, 61.7, 40.44 µg/mL, respectively. Combining lower doses of 7.5 µg/mL MPE-R or MPE-D with 7.5 µg/mL TQ decreased cell viability by 48.9% and 39.3%, respectively (Figure 4A and B), suggesting enhanced efficacy by combination treatment.

Treatment of HCT-116 cells for 72 h with 15 µg/mL of MPE-R, MPE-D or TQ decreased cell viability by 18.6, 14.3 and 26%, respectively. The IC_{50} value of each of MPE-R, MPE-D or TQ at 72 h was 33.3, 50.9, 33.5 µg/mL, respectively. Interestingly, the combination of half doses of MPE-R or MPE-D (7.5 µg/mL) with 7.5 µg/mL TQ caused a respective decrease in viability of 40.9% and 34.4% at 72 h (Figure 5A and B). Thus, combining propolis extracts with TQ enhanced their anticancer activities against breast and colorectal cancer cells.

DISCUSSION

The intake of dietary antioxidants is known to support the endogenous antioxidant system and prevent oxidative stress-mediated diseases[35]. Studies have shown that combining dietary antioxidants from different sources produces more potent antioxidant effects and possibly more effective therapeutic potential than single agents. Combining *Nigella sativa* oil with honey was shown to augment its

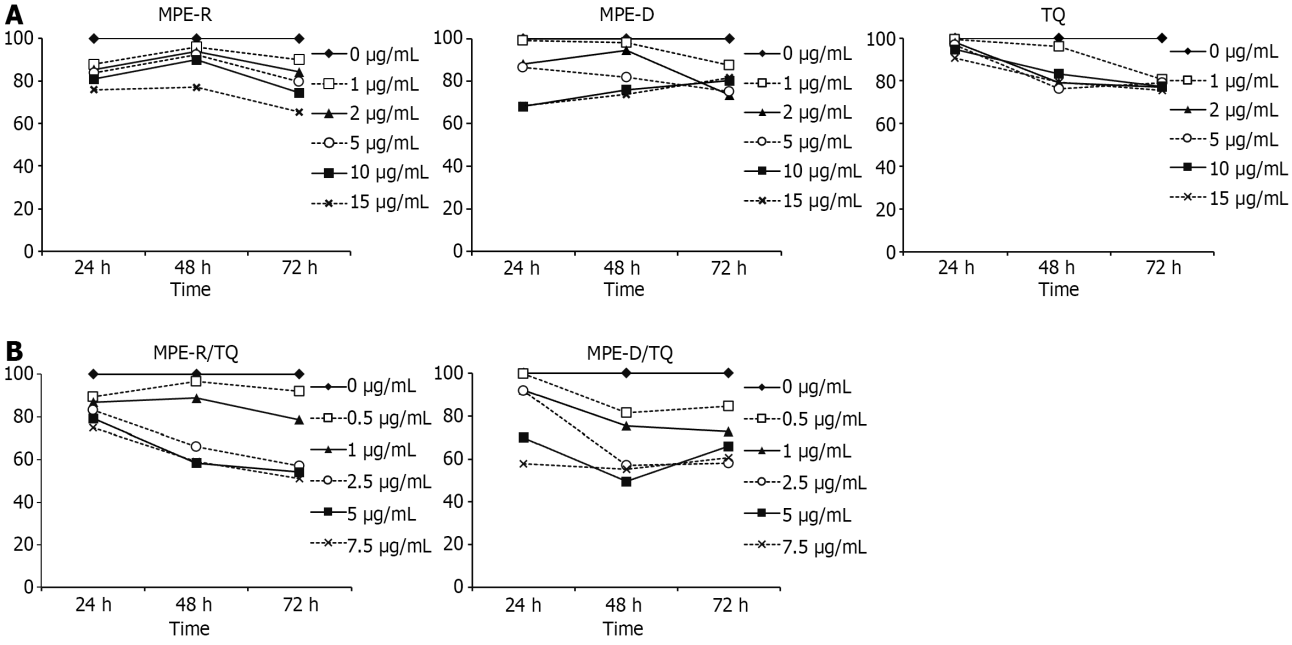

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Figure 4 Anticancer activity of Thymoquinone and methanol propolis extract from Rashaya and Akkar-Danniyeh against MDA-MB-231 human breast cancer cells. A: Cells were treated with each of methanol propolis extract from Rashaya (MPE-R), methanol propolis extract from Akkar-Danniyeh (MPE-D) and Thymoquinone (TQ; 0-15 μg/mL) alone for 24, 48 and 72 h; B: Cells were treated with the combination of each of MPEs (0-7.5 μg/mL) with TQ (0-7.5 μg/mL) for the same time point. Cell viability was then determined using MTT assay. The values are expressed as percentage of viable cells relative to untreated control. Each value represents the mean ± SD of n = 1 experiment performed in duplicates.

antioxidant capacity[36]. In addition, the combination of *Nigella sativa* seeds and honey exhibited antioxidant effects and decreased the viability of ovarian cancer cells[37]. Interestingly, oral intake of honey potentiated the protective effect of *Nigella sativa* grains against methylnitrosourea-induced oxidative stress and carcinogenesis in Sprague Dawely rats[38]. Here, we evaluated the antioxidant and the anticancer potential of combining propolis, the third most important component of bee products [39], with TQ as the major bioactive constituent of *Nigella sativa* essential oil. The key finding of the present study is that combining TQ with Lebanese propolis at half its concentration resulted in an enhanced antioxidant and anticancer effects in comparison to propolis alone as demonstrated by the improved DPPH radical scavenging activity and inhibitory effects against breast and colorectal cancer cell lines.

First, we assessed the total phenolic content of propolis collected from two different Lebanese regions Rashaya and Akkar- Danniyeh. The phenolic content is the most widely investigated among all the components of propolis because it was reported to be mainly responsible for its biological activity[40]. According to the results reported by El-Ali *et al*[41], the total phenolic content of ethanol extract of propolis collected from the two Lebanese regions Debaal and Wadi Faara were similar to our study's finding. On the other hand, higher phenolic content values were recorded in the ethanol extract of propolis collected from the Lebanese regions Fakeha and Berqayel and the citrus groves of the Lebanese coast[41,42]. This variation in total phenolic content of propolis collected from different Lebanese regions could be attributed to several factors including the botanical origin of the raw material, mode of collection, collecting season, or the solvent used in the extraction method[40].

Next, we assessed the antioxidant activity of MPE-R and MPE-D alone or in combination with TQ using DPPH free radical scavenging test. DPPH is a stable nitrogen-centered free radical which color changes from violet to yellow when it receives a hydrogen- or electron- from an antioxidant[43]. MPE-R and MPE-D exhibited significant DPPH scavenging efficacy reflecting the presence of antioxidants within their constituents. Numerous studies reported a positive correlation between antioxidant activity of propolis extracts and their contents of phenolic compounds suggesting that they are responsible of the antioxidant activity of the extracts[41,44,45]. Phenolics are known to have a hydroxyl group attached to their aromatic ring which can donate electron to free radicals and therefore stabilize them[46]. As for TQ which is a non-phenolic compound, a recent computational study reported that it attacks free radical preferentially at its 3CH position and preferably *via* hydrogen atom transfer[47].

After demonstrating the antioxidant efficacy of each of MPE-R, MPE-D and TQ, we assessed their potential to protect red blood cells from oxidative damage and hemolysis induced by H_2O_2. Red blood cells are highly prone to oxidative damage due to its high membrane concentration of polyunsaturated fatty acids[48]. When the membrane lipids of red blood cells are subjected to ROS attack, they lose a hydrogen atom from an unsaturated fatty acyl chain. This initiates lipid peroxidation that propagates as

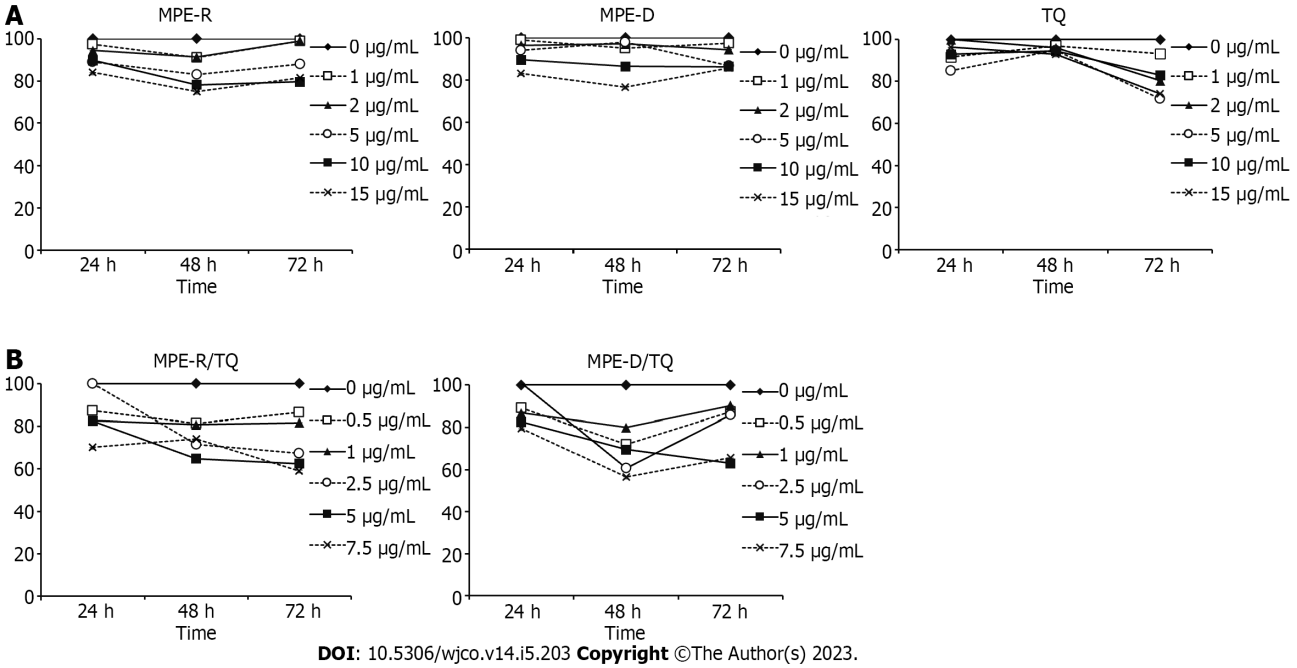

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Figure 5 Anticancer activity of Thymoquinone and methanol propolis extract from Rashaya and Akkar-Danniyeh against HCT-116 human colorectal cancer cells. A: Cells were treated with each of methanol propolis extract from Rashaya (MPE–R), methanol propolis extract from Akkar-Danniyeh (MPE-D) and Thymoquinone (TQ; 0-15 μg/mL) alone for 24, 48 and 72 h; B: Cells were treated with the combination of each of MPEs (0-7.5 μg/mL) with TQ (0-7.5 μg/mL) for the same time point. Cell viability was then determined using MTT assay. The values are expressed as percentage of viable cells relative to untreated control. Each value represents the mean ± SD of n = 1 experiment performed in duplicates.

a chain reaction and lead to membrane damage and consequently hemolysis[49,50]. Our findings are in the same line with previous research that has shown the anti-hemolytic activity of propolis or TQ under oxidative stress conditions[40,51]. The antihemolytic activity of MPE-R and MPE-D could be associated with their phenolic content. Phenolic compounds are supposed to donate electrons to hydrogen peroxide, neutralize it to water and prevent it to induce hemolysis[52].

The assessment of hemolytic activity of blood-contacting compounds is of high importance for their future application *in vivo*[53]. Our results are in agreement with those reported by Shubharani *et al*[54] who showed that low concentrations of ethanolic extract of Indian propolis did not have hemolytic activity. Although high concentrations of Lebanese propolis showed low to moderate hemolysis, same concentrations of Polish or Brazilian propolis extract did not cause hemolysis[40,55].

Cancer cells exhibit elevated levels of ROS which promote cell cycle progression and lead to an increase in cell proliferation[56]. By-products of oxidative damage such as 8-hydroxy-2-deoxy-guanosine, malondialdehyde, 4-hydroxy-2-nonenal, and carbonylated proteins were speculated to play a mutagenic role[57]. In addition, oxidative stress was found to be responsible for inactivation of several key proteins such as caspases, phosphatases, and phosphatase and tensin homologue, and inhibits p53 binding to gene promoters which reduce apoptosis and increase cell survival[58]. Dietary antioxidants have been demonstrated to have chemopreventive and anticancer effects *in vitro* and *in vivo*[59]. Numerous studies demonstrated the anticancer effect of each of TQ, propolis and its phenolic compounds in different types of cancer[60,61]. To our knowledge, this is the first study that demonstrates the promising anticancer effect of the combination of these agents. Only one study demonstrated the anticancer effect of Lebanese propolis collected from the south of the country on leukemic T cells[10]. Although MPE-D had higher antioxidant activity than MPE-R, the inhibitory effect of both extracts on the cell viability of cancer cell lines was almost the same. This result suggests that phenolic compounds may not be responsible for this inhibitory effect of the extracts.

CONCLUSION

In summary, the Lebanese propolis from Rashaya and Akkar-Danniyeh exhibited promising therapeutic potential as reflected by their potent DPPH radical scavenging activity, protective effects against H_2O_2 induced hemolysis and inhibitory effects against breast and colorectal cancer cell lines. The combination of TQ with propolis resulted in enhanced antioxidant and anticancer activities in comparison to single treatments. Thus, this combination could have potential health benefits and holds promise for the prevention of oxidative stress related diseases. Further studies should be conducted to analyze the

chemical composition of propolis, decipher the antioxidant and anticancer mechanism of its combination with TQ in addition to evaluating the effects of TQ and propolis in animal models of oxidative stress.

ARTICLE HIGHLIGHTS

Research background

Oxidative stress is implicated in the pathogenesis of numerous diseases including cancer. Propolis, the third most important component of bee products, and Thymoquinone (TQ), the main constituent of *Nigella sativa* essential oil, were extensively reported to have antioxidant and anticancer effects. However, the antioxidant potential of the combination of these natural products as well as their anticancer activity against breast and colorectal cancer cells have not been investigated yet.

Research motivation

To establish a new therapeutic approach for oxidative stress induced cancers using a combination of natural agents from food and diets.

Research objectives

To investigate the antioxidant and anticancer potential of Lebanese propolis and TQ alone and in combination.

Research methods

Folin–Ciocalteu method was used to determine the total phenolic content of the methanolic extract of propolis. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay and the H_2O_2-induced oxidative hemolysis of human erythrocytes *in vitro* assay were employed to assess the antioxidant activity of TQ and Lebanese propolis. The MTT assay was used to evaluate the anticancer activity of these natural agents in single and dual treatment against HCT-116 human colorectal cancer cells and MDA-MB-231 human breast cancer cells *in vitro*.

Research results

Combination of TQ with Lebanese propolis at half its concentration improved the antioxidant and anticancer activity of propolis as reflected by the enhanced DPPH radical scavenging activity and inhibitory effects against breast and colorectal cancer cells.

Research conclusions

Our results suggest the use of a combination of TQ and Lebanese propolis as potential therapy for the management of oxidative stress and treatment of breast and colorectal cancer. This is the first study to report the promising enhancement in Lebanese propolis antioxidant and anticancer activity when combined with TQ.

Research perspectives

Further research on the antioxidant and anticancer mechanisms of the combination of these natural agents and its therapeutic effects in animal models of oxidative stress should be performed in the future.

FOOTNOTES

Author contributions: AlDreini S carried out lab work as part of her MSc thesis, performed analysis and interpretation of data; Fatfat Z drafted the manuscript; Abou Ibrahim N provided propolis, and contributed intellectually to the study; Fatfat M supervised the experimental work; Khalife H reviewed the manuscript and contributed in the critical appraisal of data; Gali-Muhtasib H conceived the project, supervised the work, and edited the manuscript draft; All authors have read and approved the final manuscript.

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Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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