Sulphated tubers extract from the giant taro

Alocasia macrorrhiza inhibits the carcinogenesis initiation and modulates macrophage functions

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Received: 01 September 2021; Accepted: 19 December 2022

INTRODUCTION

Among complementary and alternative medicine (CAM) is investigating conventional therapeutic approaches to suppress cancer incidence, e.g., natural, semi-synthetic, or synthetic derivatives of phytochemicals to prevent or treat cancers (Rizeq et al., 2020). Carcinogenesis is an intricate phases-cascades (Kotecha et al., 2016). Tumor initiation is the first of these phases. It is fast and irreversible, as it begins with an exposure to a carcinogen and spread/transport into tissues resulted in mutations. These “initiated cells” colonize and a panel of irreversible genetic alterations occur, which persevered in the new cell generation (Barcellos-Hoff et al., 2013). Tumor promotion phase encompasses a discriminating cell-clone increase and cell division of the initiated cells permitting the accumulation of mutations. As a result, active pre-neoplastic cells start division and spreading. Finally, progression phase arises after mutation-dependent invasive cellular phenotype with a growing metastatic affinity (Kotecha et al., 2016).

Alocasia macrorrhiza (L.) G. Don, folk Araceae, is a traditionally edible plant known as giant taro, giant alocasia, biga, ape, and pia (Lim et al. 2015). Folk medicine utilizes giant taro in east Asian regions (Srivastava et al. 2012). Previous studies indicated that A. macrorrhiza polysaccharides are the main constituent in the plant tubers. The Rhizomes contain lignans, alkaloids, fatty acids, phenolics, and phytosterols (Huang et al., 2017). Generally, the chemical constituents of A. macrorrhiza are reported to have many pharmacological activities including antinociceptive, anti-fungal, anti-inflammatory, and anti-tumor activities (reviewed in Lim et al. 2015). A. macrorrhiza showed a specific treating effect in the tuberculosis, nasopharyngeal carcinoma, appendiceal abscess, and in influenza prevention. The sulphated polysaccharides...
provided cancer chemopreventive affinity (Gamal-Eldeen et al., 2007a,b; 2020).

Mycorrhizas have shown anti-cancer activity (Arbain et al., 2022) against variable carcinoma such as liver, throat and nasopharyngeal carcinoma (Zhu et al., 2012). Lei et al. (2012) confirmed that ethanolic extracts of A. macrorrhiza rhizome exhibited anti-cancer activity effect against gastric cancer cellsM-GC803 and assumed that primary biochemical compounds and secondary metabolites are responsible for this activity.

An in-vitro study on aqueous extract of giant taro revealed the suppressed proliferation and elevated apoptosis in hepatocellular SMMC-7721 cells, through expression induction in caspase-3, PPAR-γ and Bax accompanied with expression inhibition in anti-apoptotic protein (Bcl-2) and Cyclin D1 (Fang et al., 2012). In tumour grafting study the extract led to a reduction in the tumour weight was observed, without any toxicity sign, as a result of diminished DNA synthesis, stimulated arrest in G0/G1-phase, elevated apoptosis via upregulated PPAR-γ, Rh, Baz, and caspase-3 and down-regulated Bcl-2 and Cyclin D1 (Fang et al., 2012). There is a lack of studies the sulphation of A. macrorrhiza polysaccharides and no evidences to justify extract role in the tumor anti-initiation.

The current work aimed to inspect cancer chemopreventive characteristics of the sulphated tubers extract of A. macrorrhiza. Since with a special emphasis on the carcinogenesis initiation through modulating carcinogen metabolizing/detoxification enzymes. Where Tumor anti-initiation activity was investigated by estimation of SAM effect on cytochrome P450 1A1 (Cyp1A1) glutathione-S-transferases (GST), glutathione (GSH), epoxide hydrolase (mEH), and quinone reductase (QR), while Tumor anti-promotion activity was investigated by macrophage proliferation, nitric oxide (NO), and binding of LPS to macrophages.

MATERIAL AND METHODS

Characterization of sulphated A. macrorrhiza extract

A. macrorrhiza is a wide-spread in the Egyptian market as edible plant and there is no extinction risk. The tubers of A. macrorrhiza was collected from Botanic Garden, NRC, Cairo, under the permission of NRC herbarium and as identified by NRC herbarium supervisor (for voucher specimen: #2018-AM23), and according to NRC guidelines that were aligned with national and international guidelines. Tubers were washed, peeled and macerated in distilled water by a blender. Extraction performed by 1 h with hot water under reflux, and then filtered and dialyzed for 48 h against running water before being incubated in cold ethanol overnight. After centrifugation, the precipitate submitted for vacuum drying (crude polysaccharides). Sulphated A. macrorrhiza (SAM) was prepared according to previous protocols (Mährner et al. 2001; Yang et al. 2003). Characterization was performed according to previous assays (Dubois et al. 1956; Lowry et al. 1952, Partridge et al. 1949; Larsen et al. 1966; Hunt 1980).

 Culturing Cells

Human colon cancer (HCT-116), human hepatocellular carcinoma (HepG2), human acute myeloid leukaemia (HL60) and raw murine macrophages (RAW 264.7) were used (ATCC, VA, USA). Extract was endotoxins-free. Materials for cell culture were purchased from Sigma-Aldrich, USA.

Tumor anti-initiating

Inhibition of cytochrome P450 1A1 (Cyp1A1) was estimated according to Crespi et al. 1997; Gerhäuser, et al. 2003. In HepG2 cells, glutathione-S-transferases (GSTs) activity was assayed after seeding with SAM (20% of IC50, 48 h) (Habig et al. 1974). Glutathione (GSH) were detected in SAM-treated HepG2 cells (Griffith 1981). Epoxide hydrolase (mEH) activity was estimated (Inoue et al., 1993). HepG2 cytosol was submitted for quinone reductase (QR) determination (Yu et al., 2000).

Tumor anti-promoting and anti-progression activity

SAM effect on RAW 264.7 proliferation index by MTT assay (Hansen, et al., 1989). Generated nitric oxide (NO) was detected by Griess reagent in RAW 264.7 (Moorecroft et al. 2001) using bacterial lipopolysaccharide (LPS). SAM effect on the binding of macrophages to FITC-LPS was fluorometrically detected (Carracedo et al. 2002). The effect of SAM on the viability of different human cancer cells was assayed by MTT test after 48h.

Data analysis

Data statistical study was followed by Student’s unpaired t-test and one-way ANOVA test. P value was considered insignificant if > 0.05.

RESULTS AND DISCUSSION

Carcinogenesis in epithelial cells comprises successive stages: initiation, promotion, and progression. Initiation encompasses fast, direct and irreversible carcinogen/DNA binding (Tsao et al., 2004). Chemical carcinogens are usually bioactivated through their conversion into metabolites, which are electrophilically reactive and dangerous (Autrup, 1990), a step that is under the catalysis of mono-oxygenase enzymes e.g., cytochrome P-450 (Tsao et al., 2004). These metabolites may be eliminated
through enzymatic detoxification or by being conjugated to GSH, a step catalyzed by GST. Moreover, if the reactive electrophiles are epoxides, they are eliminated via epoxide hydrolase (mEH) by hydration into their corresponding trans-dihydrodiol (Tsao et al., 2004; Manson et al., 1997). As an example of epoxide inducers, dihydrodiols produced by polycyclic aromatic hydrocarbons, which undergo CYP metabolism, thereafter dangerous reactive carcinogen; dihydrodiol-epoxides are resulted in (Cannady et al., 2002).

Cancer chemoprevention is the usage of bioactive agents to suppress tumor development. The term is applied for agents that target multiple phases of carcinogenesis by blocking carcinogens to protect DNA, enhancing DNA repair, lowering cell cycle speed and cell growth. Chemoprevention concerns in the identification of promising agents, which halt the early stages of carcinogenic cell transformation (Sapienza, Issa, 2016). The successive progress of carcinogenesis cascade is largely depending on the increased drug-metabolizing enzymes and the decreased detoxification enzymes, whereas the role of the chemopreventive agents is the repair this imbalance.

The current study is a trial to prepare a modified extract of edible plant and to investigate its role in prevention carcinogenesis initiation. After the extraction of A. macrorrhiza and the further sulphation, the sulphated extract was submitted to a battery of characterizing assays, where the chemical constituents of SAM were investigated. As shown in Table 1, the findings indicated that SAM enclosed 55.2% of sulfate substitution and a degree of sulphation equals of 2.4 (molar ratio to monosaccharide unit). These results displayed that the sulfation process was sufficient. Moreover, the chromatographic analysis of acid hydrolysates of SAM affirmed the occurring of remarkable amounts of uronic acids, galactose, glucose, and mannose, in addition to traces of rhamnose, arabinose, and xylose. Most carcinogens undergo metabolic activation via Phase I enzymes that ultimately enhance their critical biological effect. This activation resulted in reactive metabolites that largely depending on cytochrome P450 enzymes metabolism. The assessment of SAM effect on Cyp1A1, a leading phase I-enzymes that activates procarcinogens transformation into actively dangerous carcinogens, showed that that the incubating of SAM (1 µg/ml) with Cyp1A1 enzyme (1 µg/ml) resulted in a potent inhibition in its activity with an inhibition percentage of 51.45% (P < 0.01), compared to the control (Fig 1a).

GSH, cytosolic thiol, is a major factor for cell retorting to oxidative stress that assists in the demolition of free radicals, lipid peroxides as well as hydrogen peroxide (Aggarwal and Shishodia, 2006). A key factor which influences homeostasis of GSH and its conjugation by GSTs, during the detoxification process to get rid of the substrates of carcinogens and xenobiotics by the catalysis of nucleophilic GSH sulfur atom (Rooseboom et al., 2004). The determination of GSH concentration in the treated HepG2 cells revealed that SAM elevated GSH, after 48 h, however the elevation was only significant (P < 0.05) in cells treated with 40 µg/ml of SAM (Fig 1b).

Increment of the levels or activities of detoxification phase II enzymes (e.g., GST, mEH and QR) represents the main protection mechanism to confront the chemical stress and therefore the carcinogenesis initiation via binding/excretion of carcinogens (Bertram, 2000). GSTs was assessed in HepG2 cells, as an important phase II enzyme after 48 h with SAM. GSTs activity increased significantly up to 162.47% of the control (P < 0.05) in cells treated with 20 µg/ml of SAM, as shown in Fig 1c. Among phase II enzymes, QR role in the reduction of electrophilic quinones is a vital detoxification pathway. After a 48 h of HepG2 cells seeding with SAM, a significantly high activity of QR (P > 0.05) was only observed at high concentrations of SAM (20 µg/ml and 40 µg/ml), (Fig 2).

The metabolic enzyme mEH adds water to arene oxides and alkene epoxides (Cannady et al., 2002). mEH is targeting the xenobiotic metabolism by its two soluble forms cytosolic and microsomal (Cannady et al., 2002). The microsomal mEH specifically metabolizes cis-substituted epoxides, whereas the cytosolic form favorably metabolizes trans-substituted epoxides. mEH showed a varied specificity to substrates and it activate/detoxify xenobiotics (Omiecinski et al., 2000). In the current work, mEH was estimated in HepG2 microsomal fraction. The results indicated a remarkable gradual increase in the activity of mEH starting from the lowest used SAM concentration (5 µg/ml; P < 0.05) up to the highest used dose (40 µg/ml; P < 0.001), (Fig 2).

### Table 1: Chemical characterization of sulphated water-soluble extract of A. macrorrhiza (SAM)

<table>
<thead>
<tr>
<th>Chemical composition</th>
<th>Value</th>
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<tbody>
<tr>
<td>Carbohydrate (%)</td>
<td>38.3</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>1.6</td>
</tr>
<tr>
<td>Sulphate (%)</td>
<td>55.2</td>
</tr>
<tr>
<td>Degree of Sulphation</td>
<td>2.4</td>
</tr>
<tr>
<td>Uronic acid</td>
<td>3.3</td>
</tr>
<tr>
<td>Galactose</td>
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</tr>
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<td>Glucose</td>
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<tr>
<td>Mannose</td>
<td>9.2</td>
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<tr>
<td>Rhamnose</td>
<td>Traces</td>
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<tr>
<td>Xylose</td>
<td>Traces</td>
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<td>Arabinose</td>
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Gamal-Eldeen, et al.
The naturally-occurring and chemically-modified phytochemicals showed a multi-targeted inhibitory effect in these early stages. For instance, they may avert carcinogens from attacking targets and provides detoxification of highly reactive molecules (Kotecha et al., 2016). Phytochemicals also enhance innate immune surveillance, e.g., antigen presenting cells, and execute the abolition of transformed cells (Bui and Schreiber, 2007; Espinoza et al., 2015). In the current study, SAM influence on the proliferation index of macrophage was investigated and the macrophage functions as the maestro antigen presenting cells were also studied. The results indicated that SAM showed a mild dose-dependent immunoproliferative induction in macrophages up to 143% of the untreated cells (Fig. 3a).

On the other hand, since dietary phytochemicals as a source of chemopreventive natural bioactives unveil antioxidative, anti-inflammatory, pro-apoptotic properties and/or anti-proliferative activities (Key, 2010). In the current study, the anti-inflammatory effect of SAM was assayed as one of macrophage functions. In the current study, bacterial LPS was exerted to stimulate inflammation cascade in RAW 264.7 macrophages, prior being seeded with SAM. NO generation in LPS-stimulated macrophages was enhanced to 30 folds of the untreated cells ($P < 0.0001$). However, the combined treatment of SAM and LPS resulted in a noticeable inhibition in this NO production down to 59% of the LPS-stimulated NO level, ($P < 0.01$), (Fig. 3b). The macrophages affinity to conjugate pathogens and tumor surface antigens represents one of the fundamental mechanistic functions occurred during macrophage activation. This conjugation was investigated after incubating macrophages in presence and absence of SAM (10 µg/ml), with FITC-LPS. The findings verified that SAM dramatically increased ($P < 0.01$) the binding affinity up to 2.47-fold, compared with that in absence of SAM (Fig. 3c). Such an activity can help in the elimination of pre-neoplastic and neoplastic cells by macrophages.

The identification of such promising chemopreventive agents in the laboratory screenings indicated that, in the cultured tumor cells, some molecules not only suppress the cell proliferation but provokes other mechanisms that regulate reversion of malignant cells into normal (Patterson et al., 2013). In our study, we screened SAM cytotoxicity against three different human cancer cell lines. The results showed that SAM was only cytotoxic against colon HCT-116 cells (IC$_{50}$ 59.6 µg/ml), as shown in Fig. 3a, whereas there exhibited a non-significant effect in both of HepG2 and HL60 cells.
CONCLUSION

SAM is suggested as a promising inhibitor of the carcinogenesis initiation phase, since it inhibited the carcinogen metabolizing enzyme Cyp1A1 and induced, to variable extent, the detoxification enzymes (GST, mEH and QR), especially mEH. Additionally, SAM showed an anti-inflammatory property and it induced the affinity of macrophage to bind pathogens and neoplastic cells. Besides tumor anti-promoting activity, SAM showed a mild cytotoxicity in colon HCT-116 cells.

CONFLICT OF INTEREST

We declare that there is no conflict of interest.

ACKNOWLEDGEMENT

This work was supported by Taif University Researchers Supporting Project Number (TURSP-2020/103). The authors gratefully acknowledge the support of the Deanship of Scientific Research, Taif University.

Author contributions

Gamal-Eldeen: Study concept/design and writing manuscript; Amer: Plant extraction, sulphation and characterization; Hawsawi, Alhuthali and Raafat: Cell-based assays; Alharthi: statistical analysis.

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Fig 3. Tumor anti-promoting activity of SAM: Sam effect on macrophage proliferation (a; green squares-line) and macrophage functions including SAM inhibition of NO production in LPS-stimulated Raw 264.7 (b) and SAM stimulation of FITC-LPS binding affinity (c). Tumor anti-progression activity of SAM: cytotoxicity was assayed HepG2, HCT-116, and HL-60 cells (a). Data are expressed as mean percentage ± S.D* P<0.05 and **P<0.01


