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Antioxidant, enzyme inhibitory and apoptotic activities of alkaloid and flavonoid fractions of *Amaranthus spinosus*

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ABSTRACT

Plants belonging to the genus Amaranthus is widely consumed by humans and animals and is known for their nutritive value and health beneficial effects. Literature indicated the potential of *Amaranthus spinosus* plants as efficient radical scavengers and chain-breaking antioxidants as well as their efficacy against inflammatory insults. It has been claimed that the phenolic compounds present in the *A. spinosus* are responsible for its pharmacological values. However, there is no clear cut information available on this matter; therefore, the study compared the crude *A. spinosus* extract and its alkaloid and flavonoid fractions as a possible chain-breaking antioxidant and cytotoxic molecules. The study observed a significantly higher ($p < 0.001$) anticancer potential for the alkaloid fraction (23.29 ± 2.19 μg/mL). Corroborating with this, the expression of apoptotic genes were significantly upregulated in the higher concentration of different extract treated cells; besides, at the same doses, the antiapoptotic genes were downregulated. The DPPH radical scavenging has been significantly high ($p < 0.001$) in flavonoid fraction (22.19 ± 3.55 μg/mL); whereas, hydrogen peroxide scavenging was found to be similar in crude and flavonoid fractions (30.22 ± 3.12 and 27.95 ± 2.71). The study thus suggests that antineoplastic properties of the plant might have contributed by the bioactive compounds in the alkaloid fraction; whereas, bioactive flavonoid components constituted the radical scavenging properties of the plant. Thus, the differential pharmacological activities of the plant may be due to the synergism between these classes of phytocompounds.

1. Introduction

Plants are the primary producers of the ecosystem and they satisfy the energy requirement for other organisms including humans [1,2]. Several plants are being included as a part of the human diet, where cereals, pulses, vegetables and fruits are predominant [3]. Vegetables form a major part of our diet by contributing essential nutrients, vitamins and minerals as well as other bioactive components required for our body [4]. Leafy vegetables are one among these, where numerous plants are being used in different parts of the world. Amaranth or Amaranthus is a common edible plant that has been consumed in various geographical regions of the world, either raw as cooked form. The plant has a high nutritional and pharmaceutical value and therefore gained much attention in the area of research [5,6].

Amaranthaceae family is renowned for their higher nutritive values and pharmacological potentials. Plants belonging to this family are reported to have strong anti-inflammatory properties, subsequently been used against diseases including arthritis [7]. *Celosia* sp. has been reported to have significant vasodialatory potentials in preclinical models and are reported to have ethnomedicinal importance also [8]. Besides, the *Amaranthaceae* plants and isolated flavonoid compounds are reported to be antimicrobial in nature [9]. Beyond their pharmaceutical capabilities, plants like Atriplex sp are also found to have significant pesticidal properties [10]. The Amaranthus plant is rich in amino acids, protein, minerals and other vitamins [5,11]. More than 50 species of Amaranthus are available, which are used as food or for non-edible purposes. Traditional medicinal systems including Ayurveda have been using the different parts of Amaranthus against different pathologic conditions. Pre-clinical and cell culture-based studies have also been carried out on the different species of Amaranthus using different extraction methods. Antioxidant properties including radical scavenging, reducing ability and metal chelating potentials have been shown by the extracts of *A. caudatus* [12], *A. tricolor* [13], *A. viridis* [14] and the bioactive carbohydrate polymer of *A. hybridus* [15]. Anti-inflammatory

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activities of different species of Amaranthus are also been described [16]. In patients with central and peripheral obesity, the oil from *A. cruentus* reduced the oxidative metabolism in neutrophils and thereby reducing inflammatory cytokine production [17]. Another study has attempted to compare the chemical characteristics and antioxidant effects of extracts prepared from different parts of various species of Amaranthus; among these, *A. hypochondriacus* was reported of having radical removal potentials and a significant correlation between phenolic content and the antioxidant activity was observed [18].

Anticancer potentials of *A. lividus* and *A. hybridus* are also been discovered against Ehlrich's Ascites Carcinoma cell lines [19]. A lectin compound isolated from the seeds of *A. viridis* inhibited the growth of two murine cancer cell lines in vitro [20]. Apart from these, the plant is well-known for its anti-diabetic [21] and also found to be protective against multiple organ toxicity [22]. The bioactive components include betacyanins, rutin, quercetin, phenolic compounds and their derivatives [23]. It is assumed that the biopharmaceutical potentials of the plant are mainly due to their phenolic compounds.

Amaranthus spinosus belonging to the family Amaranthaceae is a less studied plant for its pharmacological potentials. Literature has also indicated the antioxidant properties of *A. spinosus* extract in different models [24]. A tetraenoic fatty acid extracted and purified from the *A. spinosus* has been shown to exhibit the anti-proliferative potential and induced cell cycle arrest at the G2/M phase [25]. Polyphenols or their derivatives are thought to be responsible for the biopharmaceutical potentials of the plant [26]. However, there is no clear information is available to prove the assumption. Thus, the present study compared the radical scavenging, reducing potential and anti-proliferative properties of *A. spinosus* methanol extract; in order to see the active compounds, a comparative analysis has also been made between the alkaloid and flavonoid fractions of the plant.

2. Materials and methods

2.1. Reagents, cell culture materials and cell lines

Methyl alcohol (100%), dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and Thiazolyl Blue Tetrazolium Bromide (MTT) of reagent grade were obtained from SRL Pvt. Ltd. (Maharashtra, India). Hydrogen peroxide, RPMI-1640 Media with L-glutamine and sodium carbonate, FBS (fetal bovine serum), antibiotic cocktail, HEPES buffer and other cell culture reagents were also used (Sigma Aldrich, St. Louis, MO, United States).

A human triple-negative breast cancer cell line (MDAMB-231 cells) was cultured in a T-25 cm^2 flask. The media was replaced with complete growth media and maintained under standard cell culture conditions mentioned by previous methods of Sheela, Narayanankutty, Nazeem, Raghavamenon and Muthangaparambil [27].

2.2. Plant collection and isolation of alkaloid and flavonoid fractions

Plants of *Amaranthus spinosus* were collected from the Jizan region located in the Southwestern part of Saudi Arabia and a voucher specimen (No: 21,660) was deposited in the KSU herbarium. The aerial plant parts were cleaned and washed to remove dirt/dust particles and dried under shade conditions and powdered. The alkaloid and flavonoid fractions were isolated according to the methods of Quezada, Asencio, Del Valle, Aguilera and Gómez [28].

2.3. Screening for radical scavenging potentials

The crude Amaranthus extract and its respective flavonoid and alkaloid fractions were tested for antioxidant efficacy using standard assays. The DPPH radical scavenging assay was carried out according to the methods of Narayanankutty, Illam, Rao, Shehabudheen and Raghavamenon [29]. Scavenging of hydrogen peroxide radicals was also carried out strictly adhering to the previously described methods by Saeed, Guo, Azeem, Elshikh, Zainab, Ayaz, You, Alwahibi and Mehmood Abbasi [30]. Apart from the scavenging potentials, the reducing power on ferric compounds was estimated by the methods of Ooi, Yaacob, Rajab, Shahar and Sharif [31]. The *A. spinosus* extract was dissolved in dimethyl sulfoxide to yield concentrations ranging from 0 to 100 μg/mL. The inhibitory potential of radical scavenging and reducing was calculated in percentage of inhibition and IC50 values were also determined.

2.4. Enzyme inhibitory properties of A. spinosus

The inhibition of alpha-amylase and alpha-glucosidase, the enzyme involved in the digestion of polysaccharides and glucose was determined using the already published methodology of Patel and Ghane [32]. Tyrosinase inhibitory effect was determined as per a standard method [33]. The lipoxygenase and xanthine oxidase inhibition assays were conducted by previously described methods [6].

2.5. Anti-proliferative effect of crude extract, alkaloid and flavonoid fractions of A. spinosus

The human triple-negative breast cancer cell, MDA-MB-231, was seeded in TC-treated cell culture plates ($1x10^6$ cells/mL) and permitted to attach overnight at standard atmospheric conditions. Later the varying doses of *A. spinosus* crude extract, alkaloid or flavonoid fractions were mixed with the fresh media and added to the wells and again incubated (48 h). After that, fresh media containing 5 mg/mL of MTT was used to replace existing media in the well and the formazan crystals formed after 4 h of incubation was dissolved in DMSO based solubilizing agent and the optical density of each well was recorded at 570 nm spectrophotometrically [34].

2.6. qPCR analysis

The gene expression profile was determined by qPCR analysis; briefly, the MDA-MB-231 cells were cultivated and treated as described in section 2.5. At the end of treatment, the cells were extracted and used for cDNA synthesis using a Cell-cDNA synthesis kit. The qPCR analysis of relative gene expression was performed as per the previously published methods [35]. The details of primers used in the study have been attached as Table 1.

2.7. Statistical analysis

The results of the study are indicated in the format Mean ± SD and each experiment was carried out in three different sets with each being

Table 1
The primer sequences of various genes involved in apoptosis and anti-apoptotic genes in the human breast cancer cell, MDAMB-231 against the internal control gene β-actin.

Gene	Direction	Sequence
Caspase-3	Forward	5′-GCTGGATGCCGTCTAGAGTC-3′
	Reverse	5′-ATGTGTGGATGATGCTGCCA-3′
Caspase-7	Forward	5′- GGGCCCATCAATGACACAGA -3′
	Reverse	5′-GTCTTTTCCGTGCTCCTCCA-3′
Apaf-1	Forward	5′-TCTTCCAGTGGTAAAGATTCAGTT-3′
	Reverse	5′-TTGCGAAGCATCAGAATGCG-3′
Bax	Forward	5′-GAGCTAGGGTCAGAGGGTCA-3′
	Reverse	5′-CCCCGATTCATCTACCCTGC-3′
Blc2	Forward	5′-ACCTACCCAGCCTCCGTTAT-3′
	Reverse	5′-GAACTGGGGGAGGATTGTGG-3′
β-actin	Forward	5′-ACTACCTCATGAAGATCCTC-3′
	Reverse	5′-TAGAAGCATTTGCGGTGGACGATGG-3′

carried out in five replicas. Statistical operations were performed using the ANOVA and Tukey Kramer multiple comparison post-hoc test using GraphPad prism software ver. 7.0.

3. Results

3.1. In vitro antioxidant activity

The DPPH radical scavenging ability of *A. spinosus* crude extract was 61.18 ± 7.33 μg/mL (Fig. 1). The IC_{50} value for alkaloid fraction of *A. spinosus* did not vary significantly (56.81 ± 4.22 μg/mL); however, there observed a significant elevation ($p < 0.001$) in the radical scavenging activity of flavonoid fraction, thereby reducing the IC50 value to 22.19 ± 3.55 μg/mL.

The IC_{50} value for hydrogen peroxide (H_2O_2) scavenging assay was 30.22 ± 3.12 μg/mL for the crude extract of *A. spinosus*; the activity of flavonoid fraction was marginally higher, but not significantly varied (27.95 ± 2.71 μg/mL). The IC_{50} value for the alkaloid fraction was significantly ($p < 0.001$) lower than the crude and flavonoid fraction (78.22 ± 4.25 μg/mL) (Fig. 1).

3.2. A. spinosus and its enzyme inhibitory potentials

The inhibitory effects of *A. spinosus* extract on various enzymes associated with diabetes were estimated in terms of alpha-amylase and alpha-glucosidase inhibition. Both the enzymes are associated with the metabolism of carbohydrates and thereby contributing to diabetes. The inhibition was more prominent in the flavonoid fraction than that of the alkaloid fraction as well as crude extract of *A. spinosus* ($p < 0.01$). Likewise, the xanthine oxidase and lipoxygenase inhibition was also found to be high (Table 2) in the flavonoid fraction, compared to the crude and alkaloid fraction of *A. spinosus*. On contrary, the inhibition against the tyrosinase enzyme was found to be higher in the alkaloid fraction of *A. spinosus*, followed by flavonoid fraction and crude methanolic extract ($p < 0.05$).

3.3. In vitro anticancer activity

The anticancer activity against MDA-MB-231 cells was found to be high in the alkaloid fraction (23.29 ± 2.19 μg/mL). In comparison, the IC50 values of flavonoid fraction (81.13 ± 6.98 μg/mL) and crude methanolic extract (83.24 ± 7.21 μg/mL) was significantly less ($p < 0.001$) (Fig. 2).

To analyze the mechanism of cytotoxic effects, expression of genes related to apoptosis was evaluated by treating the cells with crude extract, flavonoid and alkaloid fraction at their respective IC50 values. There observed a significant elevation in the expression of apoptotic genes such as caspases, apaf-1 and bax. On contrary, the anti-apoptotic gene bcl2 was downregulated (Fig. 3). The expression of genes was highest in the alkaloid fraction compared to the crude extract and flavonoid fraction.

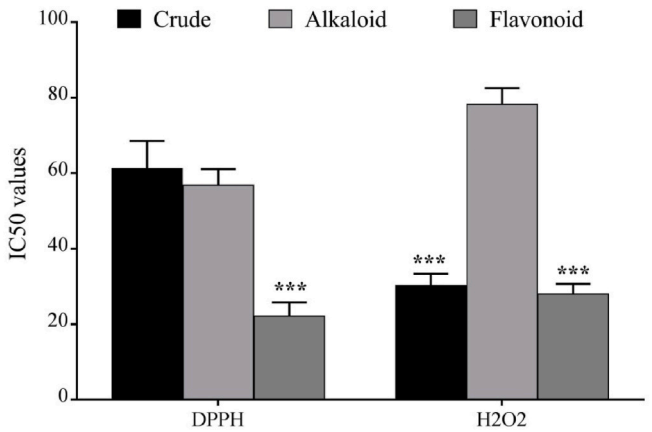

Fig. 1. The half-maximal inhibitory concentration (IC50) of crude methanol extract of *A. spinosus* along with the alkaloid and flavonoid fractions for DPPH and hydrogen peroxide scavenging.

Table 2
Antioxidant and enzyme inhibitory properties of the alkaloid and flavonoid fractions isolated from *Amaranthus spinosus*. The IC50 values are represented as mean ± SD of three independent experiments each carried out in triplicate.

Enzyme assay	Alkaloid fraction	Flavonoid fraction	Crude extract
Alpha amylase	53.2 ± 3.0	21.5 ± 1.6**	38.6 ± 2.7
Alpha glucosidase	89.7 ± 5.2	36.7 ± 2.9**	66.2 ± 3.1
Tyrosinase	18.2 ± 2.7*	28.4 ± 4.2	29.7 ± 2.1
Lipoxygenase	42.7 ± 3.8*	27.2 ± 1.8**	59.4 ± 3.3
Xanthine oxidase	79.1 ± 3.6*	76.3 ± 4.3*	98.3 ± 2.4

(* indicates significant variation at $p < 0.05$, ** indicate $p < 0.01$).

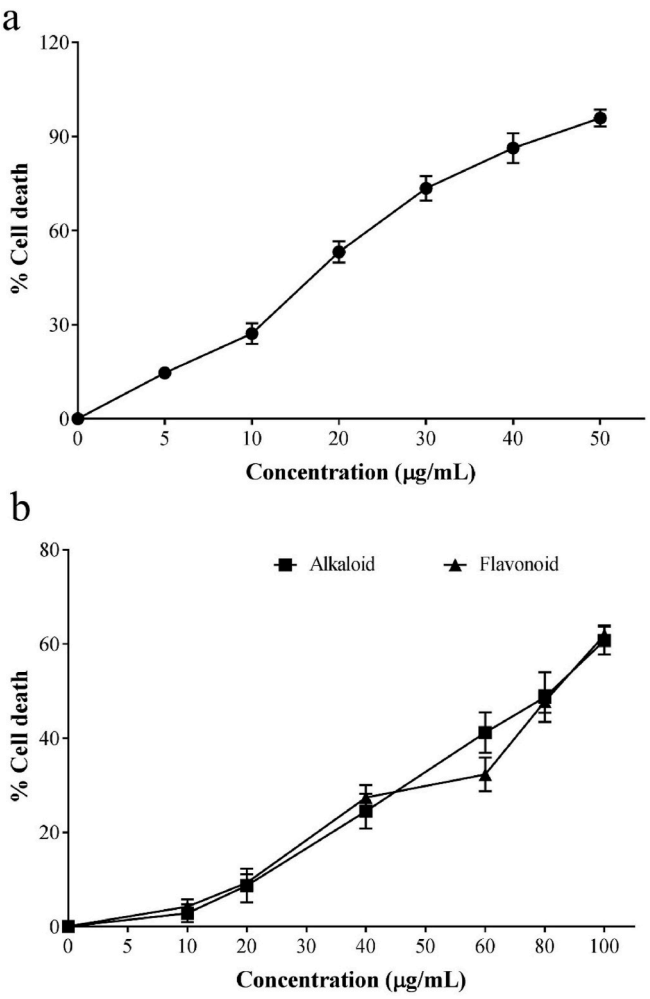

Fig. 2. The anticancer activities of crude methanol extract of *A. spinosus* along with its alkaloid (a) and flavonoid (b) fractions against human breast cancer cells (MDA-MB-231). The % cell death is plotted against the concentration of the compound (μg/mL).

4. Discussion

Vegetables are important dietary constituents that provide essential amino acids, vitamins and minerals to the body. *Amaranthaceae* family is renowned for its higher diversity and most importantly for the nutritious

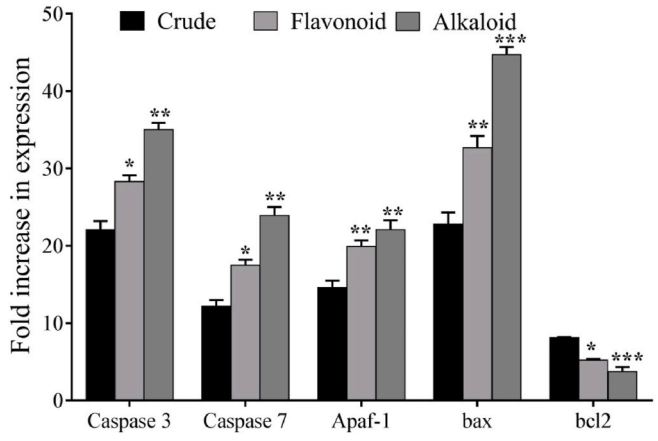

Fig. 3. Changes in the expression of apoptotic genes (caspase 3/7, apaf-1, bax) an antiapoptotic gene bcl2 in crude, alkaloid and flavonoid fractions treated at their respective IC50 value mentioned in the MTT assay.

and pharmacological values of these plants. Amaranthus is a leafy vegetable used in the daily diet of India and several other countries. The nutraceuticals properties of the plant have been already suggested by virtue of their radical scavenging, metal chelating/reducing potential and inflammation reduction abilities. Studies have previously indicated the existence of caffeic acid, ferulic acid and their derived esterified forms, and glycosides of flavonoids such as quercetin and kaempferol as well as their rutinocides [36]. Previously, studies have indicated the antioxidant activities of *A. spinosus* in various in vitro models [24]; further, it has been proposed that the polyphenol compounds are responsible for the antioxidant activities. Supporting these assumptions, the present study also observed strong antioxidant activities for the methanolic extract of *A. spinosus*, however, the flavonoid fraction has a significantly higher radical scavenging potential. On contrary, the alkaloid fraction has considerably lower antioxidant potential compared to the crude and flavonoid fraction.

The enzyme inhibitory properties of the extract have been also identified; the alpha-amylase and alpha glycosidase are enzymes associated with the metabolic conversions of carbohydrates and thereby accelerating the development of diabetes and associated complications [32]. Similarly, xanthine oxidase, which is important in nucleotide metabolism, is often an inducer of reactive oxygen species. Hence, inhibition of the enzyme is often reduced ROS levels in the cellular milieu and thereby acting as an antioxidant [37]. Lipoxygenase is an enzyme often known to initiate arachidonic acid/linoleic acid metabolism resulting in proinflammatory products such as leukotrienes [38]. It is, therefore, possible that the inhibition of these enzymes by *A. spinosus* flavonoid fraction may indicate its role in alleviating the oxidative and inflammatory insults under various diseases including diabetic complications.

The anticancer potential of the plant has been evident from the cytotoxic properties; where the alkaloid fraction has been significantly higher anticancer activity (threefold higher) than flavonoid fraction and crude extract. The increased expression of apoptotic genes such as caspases, apaf-1, and bax indicate that the mode of cell death may be apoptosis. The proapoptotic genes such as caspases and apaf-1 together with the cytochrome C results in the formation of apoptosome complex, which eventually leads to the onset of apoptotic cascades [39,40]. It is, therefore, possible that the anticancer activities of *A. spinosus* are contributed by their alkaloid fraction, rather than the flavonoids. In addition, the inhibition of the anti-apoptotic gene, bcl2, also supports this assumption. Further, reports have also indicated that plant alkaloids are strong anticancer agents [41]. The reduced cytotoxicity of *A. spinosus* crude extract and its flavonoid fraction may be due to the phytoestrogens. Flavonoids like quercetin and lignans (enterolactone and enterodiol) present in Amaranthus are reported to have estrogenic activity [42,43]. These molecules can act like estrogen receptor agonists and induce the protective/anti-proliferative properties in breast cells [44,45]. The reduced cellular expression of ERα and ERβ in MDA-MB-231 often makes these cells apt as a model for triple-negative breast cancer [46]. Influence of these phytoestrogens belonging to the flavonoid class maybe thus responsible for the reduced cytotoxicity of flavonoid fraction. In addition, studies have already reported that Amaranthus has high phenolic and flavonoid contents; therefore, the reduced cytotoxicity of crude extract may also be explained by the presence of these phytoestrogens.

The study observed significant antioxidant as well as anticancer activities for the alkaloid and flavonoid fractions of *A. spinosus*. The alkaloid fraction had higher cytotoxicity, whereas, the flavonoid portion was antioxidant-rich. It is thus concluded that the synergism between these compounds or their derivatives may be thus responsible for the pharmacological and biological properties of *A. spinosus*.

5. Conclusion

The present manuscript confirmed the radical scavenging and metal-reducing properties of *A. spinosus* and also its efficacy on enzyme inhibition and cancer cell proliferation. To provide the mode of action, the phytomolecules induced the expression of apoptotic genes and suppressed anti-apoptotic genes. Further, the antioxidant activities were higher in the flavonoid fraction, whereas, alkaloid fraction was more cytotoxic and apoptotic in triple-negative cancer cells. Overall, the study demonstrates the differential roles of individual phytocompounds in *A. spinosus* and suggests the synergy in activity during their crude form.

CRediT authorship contribution statement

Amal Al-Tamimi: Study design, Methodology approval, Data analysis. **Ahmed Alfarhan:** Methodology approval, Data analysis, Review of final draft. **Abdullah Al-Ansari:** Design, Review of Final draft. **Rajakrishnan Rajagopal:** Data collection, Analysis, Initial draft preparation. All authors reviewed and approved the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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