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A study of In vitro antioxidant and apoptotic effect of citrus medica Linn. leaves (Naarthai) against human gastric adenocarcinoma cell line (ags)

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ABSTRACT

Carcinoma or cancer of the stomach is one of the common malignancies of older men. Numerous methods of treatment options, including herbal and plant products, have been described for its management. The leaves of citrus *medica* or the naarthai leaves as it has been named traditionally do possess activities in the gastric mucosa. Hence, we decided to extract the phytochemicals from the leaves and find out the antioxidant, cytotoxic potential of the same. After confirmation of the leaves from appropriate authorities, petroleum ether, ethanolic and aqueous extracts of the leaves were made. The IC 50 was found to 200 micrograms and tested for cytotoxicity and apoptotic abilities in the AGS human gastric cell lines. The ethanolic extract showed maximal phytoconstituents. The aqueous extract showed 76.15% of scavenging effect, whereas the ethanolic extract showed 85.33% scavenging activity. Regarding the cytotoxicity effect of *C. medica* extract on the AGS cell line, it was observed that the percentage of cells was reduced to 50% in the treatment group when compared to the control group. The cells treated with ethanolic leaf extract of *C. medica* were arrested in $G_{0-}G_{1.}$ Late apoptosis was significantly increased in the treated group when compared to the control group. The ethanolic leaf extracts of citrus medica possessed antioxidant and anticancer properties in gastric mucosal lines. This option can be considered for further production of drugs from such plants.

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INTRODUCTION

Cancer is a leading cause of death in the world, and the number of such cases and deaths is expected to grow rapidly. The stomach is one of the organs prone to malignancy (Rawla and Barsouk, 2019). Most patients diagnosed with gastric cancer need chemotherapy, surgery or radiotherapy. Each of these modes has different side effects which hamper the quality of the life of the individual. Numerous chemotherapeutic agents have been used in such cases with variable rates of success. The side effects of drugs include leukopenia, anemia, tiredness, mouth ulcers, nausea, vomiting, constipation

or diarrhoea (Nurgali *et al.*, 2018). To circumvent the above problem, a few herbal drugs or chemical extracts from plants have come to the fore in different malignant settings. Veratrum californicum, Scutellaria baicalensis and Wedelia Chinensis are some of the described herbal plants with therapeutic potential against cancer (Yin *et al.*, 2013). Citrus *medica* Linn., commonly known as a Citron in English, naarthai in Tamil and bijapura in Ayurvedic literature is a shrub. Extracts of leaves of these plants have been proved to have anti-ulcer potential (Li *et al.*, 2019). Hence, we wanted to investigate the possible role of extracts of this plant in combating gastric cancer through cell line studies. The study aimed to extract the phytoconstituents from the leaves of the above-said plant and study their effects as an antioxidant, their apoptotic and cytotoxic potential in AGS gastric cancer cell lines.

MATERIALS AND METHODS

The fresh leaves of Citrus *medica* Linn. were collected in November 2018 from the local areas of Kumbakonam, Tamilnadu, India. The plant was identified and authenticated by Department of Botany, Government Arts and science college (Autonomous), Kumbakonam. The collected leaves of Citrus *medica* Linn. were washed and shade dried for two to three weeks. The dried samples were powdered using an electrical blender.

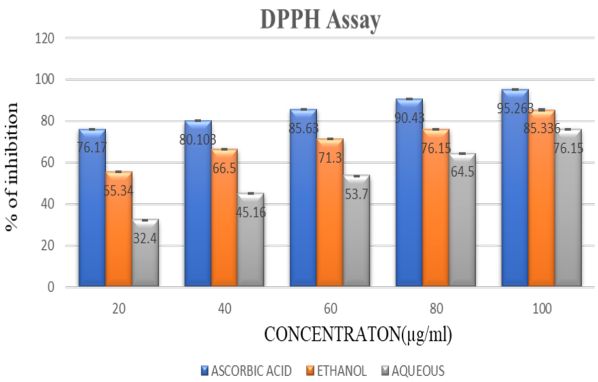

Figure 1: Effect of Citrus *medica* leaf extracts on DPPH radical scavenging activity.

Then according to classical described techniques, ethanolic, petroleum ether and aqueous extracts of the same were prepared. Phytochemical screening was done to detect alkaloids, flavonoids, saponin and steroids. The antioxidant activity was analyzed with Ferric iron-reducing antioxidant power assay (FRAP). The radical scavenging activity was done by 2,2DPPH (2, 2-Diphenyl-picryl hydrazyl) scavenging assay (Parthasarathy, 2020). The human gastric cancer AGS cell line was purchased from National Centre for Cell Science (NCCS), Pune, India. The cell count was done, and the cell viability was tested by trypan blue dye exclusion test using a hemocytometer. The cell lines were treated with different concentrations of the plant extract ranging from 25 to 400 μg for varying periods (6 hours, 12 hours,18 hours,24 hours, 36 hours and 48 hours) and the dose and time of treatment was optimized in each cell line using MTT (4, 4-dimethyl-2-tetrazolyl)-2, 5-diphenyl-2, 4-tetrazolium salt) assay. The following table Table 1 describes the group with a concentration of citrus leaves extracts.

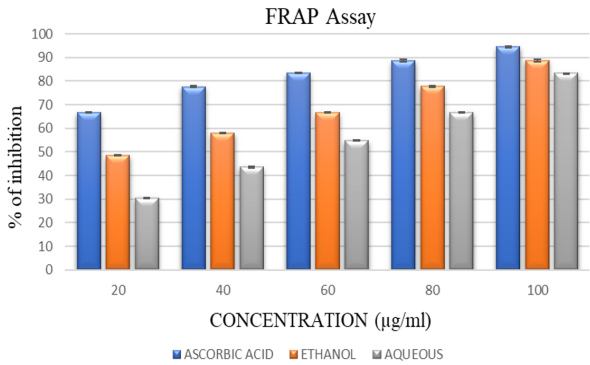

Figure 2: Antioxidant activity of Citrus medica leaf extract by Reducing Power assay.

The extent of cytotoxicity was done with sulphorhodamine B (SRB) assay (Parthasarathy, 2020). The cell cycle analyses and the detection of apoptosis were done with flow cytometry according to described protocols. All tests were repeated in triplicate to confirm the findings and establish our results.

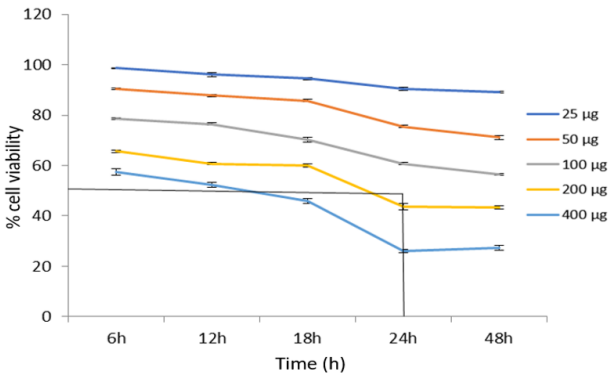

Figure 3: IC 50.

RESULTS AND DISCUSSION

The phytoconstituents in different types of extracts is tabled below in Table 2. It can be inferred from the table that the ethanolic extract of the leaves had more phytoconstituents than the other ones. The effect of Citrus *medica* leaves on radical scavenging

Table 1: Groups with a concentration of extracts.

Groups	Concentration of Extracts
Group I	Control (Untreated AGS cell)
Group II	AGS Cells + Citrus medica Leaves (25 μg/mL)
Group III	AGS Cells + Citrus medica Leaves (50μg/mL)
Group IV	AGS Cells + Citrus medica Leaves (100μg/mL)
Group V	AGS Cells + Citrus medica Leaves (200μg/mL)
Group VI	AGS Cells + Citrus medica Leaves (400μg/mL)

Table 2: Phytoconstituents.

Secondary Metabolites	Petroleum Ether Extract	Ethanolic Extract	Aqueous Extract
Carbohydrate	-	-	+
Protein	-	-	+
Alkaloids	+	+	+
Flavonoids	+	+	+
Steroids	+	+	-
Glycosides	+	+	-
Tannins	-	+	-
Phenol	-	+	-
Saponins	-	-	-

Table 3: Effect of Citrus medica extract on the distribution of apoptotic cells in the AGS cell line.

Cell population	Live cells %		Early apoptotic cells %		Late apoptotic cells %		Necrotic cells %	
	Control	Treated	Contro	Treated	Contro	Treated	Control	Treated
AGS	98.01	2.26	0	0.23	0.16	92.83	1.83	4.68

activity (DPPH) was examined at different concentrations of various extracts (aqueous, and ethanol). Figure 1 represents the scavenging activity of Citrus *medica* leaf extracts in percentage. The aqueous extract showed 76.15% of scavenging effect, whereas the ethanolic extract showed 85.33% scavenging activity. Ascorbic acid was used as standard. The scavenging effect was more with ethanolic extract, which coincides with the constituents. The FRAP assay Figure 2 revealed the ethanolic extract (88.68%) of *C. medica* showed higher reducing activity compared to aqueous extract (83.34%). This measures the antioxidant power of the extracts. IC 50 was found to be 200 micgm Figure 3. Figure 4 represents the result of cytotoxicity effect of *C. medica* extract on the AGS cell line. It was observed that the percentage of cells was reduced to 50% in the treatment group when compared to the control group. It indicates that the ethanolic leaf extract of *C. medica* showed a cytotoxic effect in the AGS cell line. The untreated cell used as the control. Concerning the apoptotic detection of AGS cell line at 24 hours exposure of 175μg/ml dosage of *C. medica* leaf extract, the cells treated with ethanolic leaf extract of *C. medica* were arrested in $G_{0-}G_{1.}$ In the early apoptosis, the percentage of cell death was slightly increased in the treated group when compared to the control group.

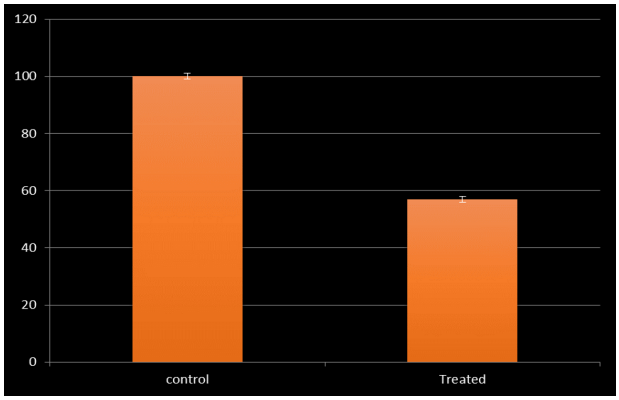

Figure 4: Cytotoxicity of ethanolic leaf extract of Citrus *medica* on AGS cells by SRB assay.

In late apoptosis, the percentage of cell death was significantly increased when compared to the con-

trol group. The results indicated that the ethanolic leaf extract of *C. medica* induces apoptosis in AGS cell line. The distribution of apoptotic cells with a comparison of both treated and untreated groups is tabled in Table 3. (Panara *et al.*, 2012) reported that the ethanolic leaf extract of *Citrus medica* contains flavonoids, alkaloids, steroids, glycosides, and phenolic compounds. Our findings go along with their results. Many authors have described the antioxidant properties regarding the antioxidant properties of the plant in general. The specific extracts of the leaves of citrus *medica* linn have been done only by a very few. (Cirmi *et al.*, 2017) have described the various constituents and extracts of citrus juices and demonstrated the anti-cancer potential. Still, the leaf extracts were not noted in their study. As the leaves (naarthai leaves) have been traditionally used for gastric ulcer problems by this part of the continent, we chose the leaf extract and stomach cancer cell lines. The leaf extracts have been found to have antihelminthic and estrogenic (Bairagi *et al.*, 2011) properties. A demonstrable anti peptic ulcer activity and thereby influencing the malignant transformation of a peptic ulcer has so far not been published. Even though the citrus peels and other fruit part extracts have demonstrable medicinal values (Song *et al.*, 2015), this study is the first of its kind to demonstrate the citrus *medica* linn leaves in gastric cancer AGS cell lines. Even though the leaf extract induces early apoptosis, it's not much significant. Late apoptosis is very significant in our study. As apoptosis (Wong, 2011) assumes significance in the discovery of novel drugs countering cancer, our study may throw light on the leaf extracts of citrus *medica* linn which may play a role in the fight against gastric cancer.

CONCLUSIONS

The results of the present study demonstrated the presence of alkaloids, flavonoids, steroids, glycosides, and phenolic compounds in the ethanolic extract of Citrus *medica (naarthai)* leaves. Antioxidant activity of ethanolic stem extract of Citrus medica leaves was confirmed by DPPH and FRAP assay. The antioxidant potential is due to the phytochemical constituents present in the leafy aerial parts of the plant. Cytoprotection was measured by cleavage of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) by surviving AGS cells following exposure to ethanolic extract of *C. medica*. The IC_{50} value was found to be 200μg/ml by MTT assay. Flow cytometry analysis concludes that *citrus medica* mediated apoptosis and induced G_0-G_1 phase cell cycle arrest only in cancer cells.

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The authors declare that they have no funding support for this study.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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