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PHYTOCHEMICAL ANALYSIS AND EVALUATION OF *IN-VITRO* ANTIOXIDANT ACTIVITY OF CLASSICAL SIDDHA HERBAL FORMULATION '*THURINJI MANAPAGU*' (*CITRUS MEDICA* SYRUP)

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ABSTRACT: Siddha science is an ancient way of healing which uses plants, minerals and metals for medicinal preparation. Research on herbs is gaining significant interest for discovering new drug molecules. In this context, it is essential to investigate the numerous traditional formulations documented in classical Siddha literature. The *Thurinji manapagu* contains *Citrus medica* and *Saccharum officinarum* is a traditional herbal syrup formulation which is indicated for nausea and vomiting, hypertension in clinical practice of siddha. The phytochemical analysis and free radical scavenging effect of the *Thurinji manapagu* was studied in this paper to explore the antioxidative efficacy of the drug. The qualitative phytochemicals of *Thurinji manapagu* was determined by standard protocols. The scavenging effect against the free radicals of the drug *Thurinji manapagu* was measured by the following *in-vitro* methods: DPPH radical scavenging assay, H2O2, ABTS, NO scavenging assay and studied through U.V spectrophotometer. The phytochemical screening of drug reveals the presence of phenols, tannins, saponins, coumarins etc. The results of the antioxidative capacity of the drug on all four assays show dose dependent and IC50 values are obtained by linear regression analysis by using percentage inhibition and concentrations. It is concluded, *Thurinji manapagu* possesses an antioxidative capacity and hence this can be used as an adjuvant supplementation for numerous pathological state which is associated with oxidative stress.

INTRODUCTION: The pharmacology of Siddha medicine encompasses nature and its resources for drug formulations. The classical literature of *Siddha pharmacopeia* has enumerated numerous drug formulations made of herbal, herbomineral, herbomarine and herbometallic preparations. The standardisation of every drug is indeed a routine procedure since this reflects the quality, purity and efficacy of drugs.

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To some extent, the concept of standardisation exists earlier in the literature of *Siddha pharmacology* (*Gunapadam*) utilizing physical parameters. The traditional textbook of *Siddha Gunapadam* mentioned different types of standardisation methods physically such as the colour of a drug, consistency of a drug, solubility and insolubility of a drug in water etc., for various forms of drug.

The modern technique which involves physical, chemical, and biological way of standardising the drug can give us more knowledge about identifying the sterileness and adulterated herbs as well as the presence of phytochemicals. The phytochemicals, secondary metabolites present in plants can be

identified with the help of chemical standardisation and are said to have therapeutic potential which ameliorate the diseases. Therefore, the science behind these ancient formulations must be thoroughly studied using both the indicators mentioned in Siddha literature and modern technologies. This approach will help document our rich healthcare heritage in a more scientific and systematic manner.

Antioxidants are now being used as a supplement for almost every health disorder because it is believed that reduced levels of antioxidants in the body could accelerate many pathologies. Despite the availability of synthetic antioxidants, research towards plant based free radical scavengers increases owing to their lesser adverse effects, cost-effectiveness and many chronic disorders may have a multifactorial origin hence the use of complex chemicals will be helpful rather using single extracts or bioactive agents [1].

Thurinji manapagu, a traditional herbal formulation of Siddha medicine is extensively used for ailments predominant with *pitha*. It is a common anti-emetic drug prescribed in the clinical practice of Siddha [2]. This *Citrus medica* juice has been used in the antenatal period traditionally to subside nausea and vomiting which is a very common manifestation in the first trimester of pregnancy [3]. The ingredients in the manapagu are the fruit part of Thurinji (*Citrus medica*) and *Sugar* (*Saccharum officinarum*) [2]. The phytochemical screening of *Thurinji manapagu* is necessary to explore the bioactive compound which could be responsible for the healing activities. This study is about the analysis of qualitative phytochemicals and ensuring the presence of antioxidants in *Thurunji manapagu* to fill up the existence of knowledge gap in traditional publications.

MATERIALS AND METHODS:

Sampling of Material: *Thurinji manapagu*, the material to be tested was obtained from the Earth India Naturals pharmaceutical; Tiruvanamalai, the Siddha drug manufacturers in Tamil Nadu, India.

Phytochemical Analysis: The qualitative phytochemical analysis was done to identify the secondary metabolites of *Thurinji manapagu* according to standard procedures [4].

Test for Alkaloids (Mayer's Test): 2ml of Mayers reagent was added to the test drug and the formation of a dull white precipitate reveals the presence of alkaloids.

Test for Coumarins: 1ml of 10% sodium hydroxide was added to the test drug and the formation of yellow colour indicates the presence of coumarins.

Test for Saponins: 5ml of water was added to the test drug and the test tube was shaken vigorously. The presence of saponins is indicated by the copious lather formation.

Test for Tannins: Ferric chloride was added to the test drug and the formation of dark blue or greenish black colour indicates the presence of tannins.

Test for Glycosides (Borntrager's Test): To identify the presence of glycosides in the test drug, it is hydrolysed with concentrated hydrochloric acid for 2 hours in a water bath, and the filtered hydrolysate is obtained. Now, to the 2ml of filtered hydrolysate, 3 ml of chloroform was added and shaken, chloroform layer is separated and 10% ammonia solution is added. The pink colour indicates the presence of glycosides.

Test for Flavonoids (Alkaline Reagent Test): To 2ml of the test drug, two to three drops of sodium hydroxide were added. There is a formation of deep yellow colour which gradually disappears and becomes colourless by adding a few drops of dilute Hcl which indicates the presence of flavonoids.

Test for Phenols (Lead Acetate Test): 3ml of 10% lead acetate was added to the test sample and the formation of a bulky white precipitate denotes the presence of phenolic compounds.

Test for Steroids: A few drops of concentrated sulphuric acid (3ml) with the 2ml of chloroform was added to the test drug and the test tube was shaken. The test tube shows the red color in the upper layer and the sulphuric acid layer shows yellow with green fluorescence which denotes the presence of steroids.

Triterpenoids (Liebermann–Burchard Test): A few drops of acetic anhydride were added to the

chloroform. To the sides of the test tube, 1ml of concentrated sulphuric acid was added and found the red ring appearance which indicates the presence of triterpenoids.

Test for Cyanins (Anthocyanin): To the test sample, 1ml of 2N sodium hydroxide was added and the mixture was heated at 100°C for 5 min. The presence of anthocyanin was confirmed by the formation of a bluish green color.

Test for Carbohydrates (Benedict's Test): The mixture of 0.5 ml of Benedict's reagent and the test drug was heated in a boiling water bath for 2 minutes and the characteristic coloured precipitate represents the presence of sugar.

Proteins (Biuret Test): 1% solution of copper sulphate followed by a 5% solution of sodium hydroxide was added to the test drug and the proteins presence was identified with the formation of a violet purple colour.

***In-vitro* Antioxidant Assay:** The radical scavenging activity of the sample has been performed by the following four methods: DPPH, NO, ABTS, and Hydrogen Peroxide radical scavenging assay [5-8].

DPPH (2, 2-Diphenyl -1- picrylhydrazyl) Assay: To determine the presence of antioxidant activity in the test drug sample *Thurinji manapagu* (TUM), 2,2-diphenyl -1- picrylhydrazyl (DPPH) free radical scavenging assay was used. The concentration of 10-100 µg/ml of Sample TUM, along with standard ascorbic acid was employed for detection of DPPH scavenging activity. The reaction mixture was prepared by adding 1 ml of 0.3mM DPPH methanol solution to 2.5 ml of sample TUM at various concentrations at room temperature. Absorbance at different concentrations of sample TUM (10 µg, 20 µg, 40 µg, 60 µg, 80 µg and 100µg/ml) was measured after 15 min incubation period at 37^0C. The absorbance was recorded at 517 nm using a double-beam UV Spectrophotometer, with methanol used as the blank. The radical scavenging is determined by the formula:

$$\text{Radical scavenging (\%)} = (A)_{control} - (A)_{sample} / (A)_{control} \times 100$$

The IC_{50} value, which represents the effective concentration of test sample TUM required to scavenge 50% of the DPPH radicals, was determined by performing a linear regression analysis of dose-response curve plotting between % inhibition and concentrations.

Nitric Oxide Radical Scavenging Assay: The concentrations of test sample TUM were prepared by serial dilutions ranging from 10−100 µg/mL, and gallic acid was used as the standard. Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid with 0.1% naphthylethylene diamine dihydrochloride in 2.5% phosphoric acid just before use. A volume of 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was combined with 1 mL of the test drug at various concentrations (10−100 µg/mL) and incubated at 25°C for 180 mins. After incubation, an equal volume of freshly prepared Griess reagent was added to the test drug TUM. Control samples, containing buffer instead of the test drug, were prepared in the same manner as the test samples. The absorbance was measured at 546 nm using a Spectra Max plus UV-Vis microplate reader (Molecular Devices, GA, USA). Gallic acid served as the positive control. The percentage inhibition of nitrite radical scavenging activity for both the test drug TUM and standard was calculated and recorded using the following formula:

$$\text{Nitric oxide scavenging (\%)} = (A)_{control} - (A)_{test} / (A)_{control} \times 100$$

ABTS Assay: The ABTS assay was conducted to assess the anti-oxidant potential of test drug TUM against ABTS radicals (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid). The ABTS radical cation method, which was modified to evaluate the free radical-scavenging effect of various (one hundred) pure chemical compounds. The ABTS reagent was prepared by combining 5 mL of 7 mM ABTS with 88 µL of 140 mM potassium persulfate. The mixture was kept in the dark at room temperature for 16 h to facilitate free radical generation after which it was diluted with water (1: 44, v/v). To assess the scavenging activity, 100 µL ABTS reagent was mixed with 100 µL of the test sample at the concentration of 10-100µg/ml in DD water, and was incubated at room temperature for 6 min. After incubation, the absorbance was measured 734 nm. 100% methanol was used as a control.

Gallic acid, prepared at the same concentrations as the test drug TUM, measured using the same procedures and served as a positive control. The antioxidant activity of the test sample TUM was calculated using the following equation to determine the ABTS scavenging effect.

$$\text{Radical scavenging (\%)} = (A)_{control} - (A)sample / (A)_{control} \times 100$$

Hydrogen Peroxide Radical Scavenging Assay: A hydrogen peroxide solution (2 mM) was prepared in 50 mM phosphate buffer (pH 7.4). Aliquots (0.1 mL) of the test sample TUM at varying concentrations (ranging from 10-100µg/ml) were added to the test tubes and their volumes was adjusted to 0.4 mL with 50 mM phosphate buffer (pH 7.4). After adding 0.6 mL of the hydrogen peroxide solution, tubes were vortexed. The absorbance of the hydrogen peroxide was measured at 230 nm after 10 minutes, using a blank as a reference. BHA was used as the positive control. The percentage inhibition of the test drug TUM and standard was calculated and recorded. The percentage radical scavenging activity of the test drug TUM and BHA was determined using the following formula:

$$\text{Radical scavenging (\%)} = (A)_{control} - (A)_{sample} / (A)_{control} \times 100$$

RESULTS AND DISCUSSION: This study scrutinized about phytochemical analysis and antioxidative efficacy of Siddha drug *Thurinji manapagu*. **Table 1** illustrates the phytochemicals analysis of the test drug *Thurinji manapagu* and the antioxidative capacity of test drug *Thurinji manapagu* by the following assay has been mentioned in **Fig. 1, 2, 3** and **4**. **Table 2** shows IC_{50} values obtained by linear regression analysis.

TABLE 1: PHYTOCHEMICAL ANALYSIS OF *THURINJI MANAPAGU*

S. no.	Test	Observation
1.	Alkaloids	-
2.	Flavanoids	-
3	Glycosides	+
4.	Steroids	+
5	Triterpenoids	+
6	Coumarin	+
7.	Phenol	+
8.	Tannin	+
9.	Protein	-
10.	Saponins	+
11.	Sugar	+
12.	Anthocyanin	-
13.	Betacyanin	+

From the phytochemical analysis it is evident that the drug were shown positive for glycosides, steroids, triterpenoids, coumarins, phenol, tannins, saponnins and betacyanins. The individual chemical constituents itself, exhibits antioxidant activity. The coumarin which is present in the drug are a natural phenolic compound which not only exhibits antioxidant activity but also various pharmacological actions such as anti cancer, anti inflammatory etc [9, 10]., Nadia Benedetto *et al.*, conducted a systematic review about phytochemicals constituents of *Citus medica* and the study revealed that coumarins 5,7 dimethoxy coumarin (Citropten), 6,7 dimethoxy coumarin (Scoparone) and Bergapten were derived from the plant [11]. Among these, citropten inhibits vascular smooth muscle cell proliferation and migration (responsible in development of Cardiovascular diseases including ischemic heart disease, myocardial infarction, stroke, hypertension) by activating TRPV1 (Transient receptor potential vanilloid type 1) channel [12]. Similarly, o-coumarin was the main phenolic compound from the pulp of *Citrus medica* which is said to have highest antioxidative capacity [13].

Antioxidant Assay Results:

DPPH Radical Scavenging Assay (DPPH Assay): The antioxidant activity of the sample drug on DPPH reaction shown in **Fig. 1.** The free radical scavenging activity of sample TUM exhibits dose dependent increase in percent inhibition. The highest percent inhibition exhibited by the sample was 29.33 ± 4.937 at the concentration of 100 µg/ml. The reduction of DPPH to DPPH-H causes the disappearance of the purple colour indicating the presence of antioxidant in the sample drug. The IC_{50} value obtained for the sample and the ascorbic acid (standard) was shown in **Table 2.**

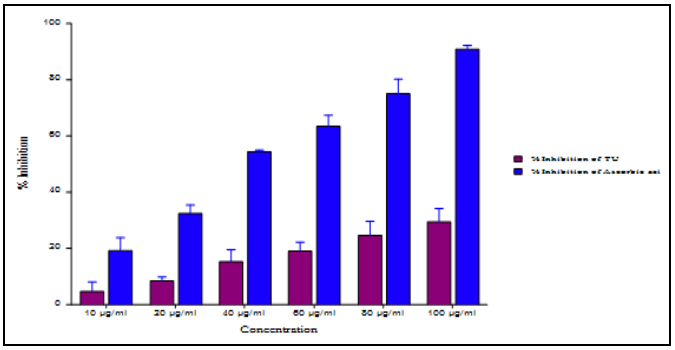

FIG. 1: PERCENTAGE INHIBITION OF TEST DRUG *THURINJI MANAPAGU* ON DPPH ASSAY

Nitric Oxide Radical Scavenging Assay (NO Assay): The formation of colorless compound during this reaction indicates the presence of antioxidant in the sample tested. This could be due to prevention of oxidation of nitric oxide to nitric ions. There noted increase in percent inhibition of test drug from 5.016 ± 1.791 to 22.01 ± 1.225 with the increase in concentration from 10 µg/ml to 100 µg/ml, which is shown in **Fig. 2.** The IC_{50} value of test drug was higher when compared to standard agent gallic acid shown in **Table 2**.

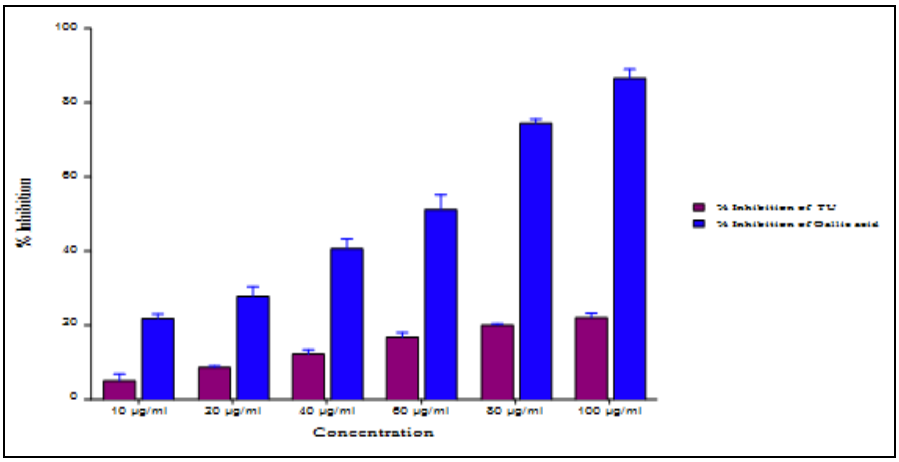

FIG. 2: PERCENTAGE INHIBITION OF TEST DRUG *THURINJI MANAPAGU* ON NO SCAVENGING ASSAY

ABTS Assay: The free radical scavenging activity of *Thurinji manapagu* on ABTS assay increases with increase in concentration of drug as shown in **Fig. 3. Table 2** shows the IC_{50} value obtained for both test drug and the standard.

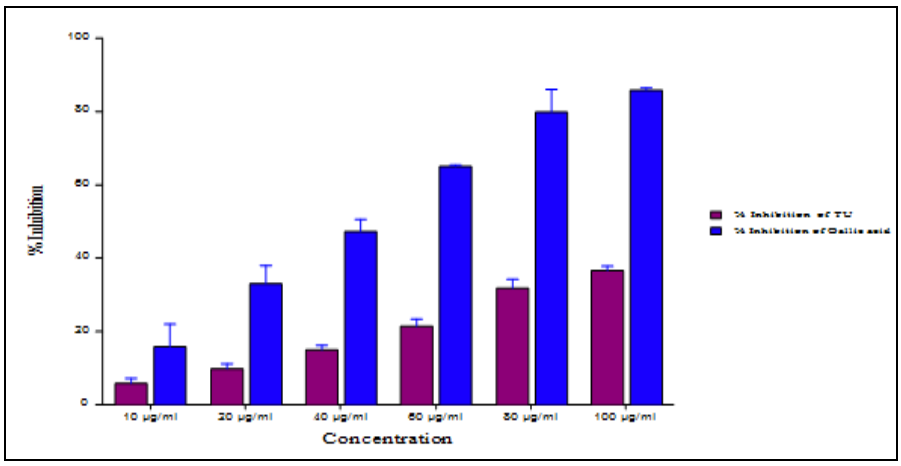

FIG. 3: PERCENTAGE INHIBITION OF TEST DRUG *THURINJI MANAPAGU* ON ABTS ASSAY

Hydrogen Peroxide Radical Scavenging Assay (H_20_2 Assay): The scavenging effect of *Thurinji manapagu* against hydrogen peroxide radical scavenging assay was concentration-dependent (10 -100 µg/ml). The percent inhibition of both test drug and Standard was shown in **Fig. 4**. The IC_{50} values for hydrogen peroxide radical scavenging assay by both test drug and standard was shown in **Table 2.**

TABLE 2: IC_{50} VALUES OF TEST DRUG *THURINJI MANAPAGU* AND STANDARD

	DPPH		NO		ABTS		H202	
IC50	**TUM**	**AA**	**TUM**	**GA**	**TUM**	**GA**	**TUM**	**BHA**
	176.8 ±27.04	44.13± 2.981	244.4 ± 24.8	44.27± 8.246	137.8 ± 6.179	45.95± 0.5081	259.3± 52.31	54.18± 12.65

Note: IC_{50} values were expressed as Mean ± SD. TUM -*Thurinji manapagu*, AA - Ascorbic acid, GA - Gallic acid, BHA - Butylated hydroxyanisole, DPPH - 2,2-diphenyl -1- picrylhydrazyl, NO – Nitric oxide, ABTS - 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), H2O2 – Hydrogen peroxide.

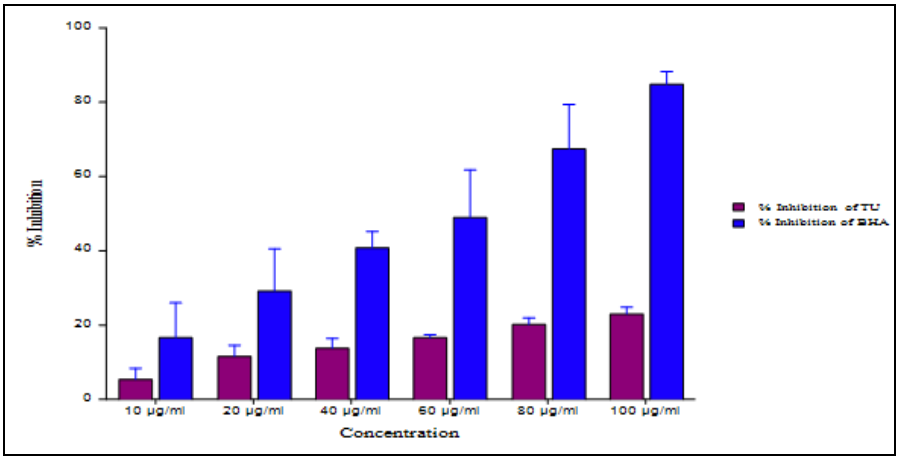

FIG. 4: PERCENTAGE INHIBITION OF TEST DRUG *THURINJI MANAPAGU* ON H_20_2 ASSAY

From the above assays, it is evident that *Thurinji manapagu* has the efficacy to protect against free radicals. Free radicals or Oxidants in turn responsible for numerous pathologies which includes diabetes, cancer, cardiovascular diseases etc [14]., Furthermore, there are few clinical trials in which anti cancer and cardioprotective nature of *Citrus medica* were studied.

In an *in-vitro* experiment conducted by Maliheh Entezari *et al., Citrus medica* (half ripe) fruit juice has been added to human astrocytoma cancer cells to study about its antimutagenicity and anticancer effects and there observed severely repressed division of cancerous cells and prevented the reverted mutation [15]. In another study, an ethanolic extract of *Citrus medica* fruit was administered to male albino rats for 15 days. On the 14th and 15th days, cardiomyopathy was induced using isoproterenol, and subsequent changes were observed. The results showed a decrease in serum levels of total cholesterol, triglycerides, and low-density lipoprotein, along with an increase in high-density lipoprotein. There was also significant attenuation in malondialdehyde (MDA) levels. Histopathological examination revealed only mild injury to the myofibrils [16]. On summary, the drug *Thurinji manapagu* which is proved to be an antioxidant has been prescribed for hypertension, vomiting especially during pregnancy (oxidative stress plays major role in both pathologies) in Siddha practices. The occurrence of prognosis in the above stated condition could be attritubed due to its potent antioxidant capacity. However, this drug needs a profound research regarding exact phytochemicals which is responsible for free radical scavenging and its molecular mechanicm towards the activity should be focussed for future research.

CONCLUSION: The presence of phenol and coumarins on phytochemical screening proves the scavenger effect of a drug. The phytochemical screening and scavenging effect of free radicals from the drug should be tested with chromatography and bioassay to know more about the bioactive compounds responsible for pharmacological actions. In addition, there needs more experimental studies and clinical trials to prove its efficacy and also evaluate its long term toxicity effects.

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REFERENCES:

1. Vaou N, Stavropoulou E, Voidarou CC, Tsakris Z, Rozos G, Tsigalou C and Bezirtzoglou E: Interactions between medical plant-derived bioactive compounds: focus on antimicrobial combination effects. Antibiotics (Basel) 2022; 11(8): 1014.
2. Mudhaliar KKN, Utthamarayan KS and Thirattu SV: (Indian medicine and Homeopathy) 2006; 259.
3. Darvishpor S, Hosseini A, Davood A, Salehifar E, Akbari J & Azadbakht M: A review on medicinal plants used for nausea and vomiting in persian medicine. Global Journal of Medical Research (B) 2018; 18(1): 29-45.
4. Brain KR & Turner TD: The Practical Evaluation of Phytopharmaceuticals, (Bristol: Wright Scientechnica) 1975; 36-45.
5. Sivaraman Dhanasekaran & Panneerselvam Perumal: *In-vitro* Screening for acetylcholinesterase enzyme inhibition potential and antioxidant activity of extracts of *Ipomoea*

aquatica Forsk: therapeutic lead for Alzheimer's disease. J of Applied Pharmaceutical Science 2015; 5(02): 12-16.
6. Panda BN, Raj AB, Shrivastava NR & Prathani AR: The evaluation of nitric oxide scavenging activity of *Acalypha indica* Linn Root. Asian Journal Research Chemistry 2009; 2(2): 148–150.
7. Pellegrini N, Re R, Ying M & Rice-Evans C: Screening of dietary carotenoids and carotenoid-rich fruits extract for antioxidant activities applying 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assay. Methods in Enzymology 1999; 299(34): 374–389.
8. Ruch RJ, Cheng SJ & Klaunig JE: Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis 1989; 10(6): 1003–1008.
9. Anjali Rawat and A. Vijaya Bhaskar Reddy: Recent advances on anticancer activity of coumarin derivatives. European J of Med Chemistry Reports 2022; 5: 100038.
10. Batoul Rostom, Racha Karaky, Issam Kassab and Maité Sylla-Iyarreta Veitía: Coumarins derivatives and inflammation: Review of their effects on the inflammatory signaling pathways. Europ J of Pharma 2022; 922: 174867.
11. Benedetto N, Carlucci V, Faraone I, Lela L, Ponticelli M, Russo D, Mangieri C, Tzvetkov NT and Milella L: An Insight into *Citrus medica* Linn.: A Systematic Review on Phytochemical Profile and Biological Activities. Plants 2023; 12(12): 2267.
12. Hoa, Pham, Trang, Nguyen Minh, Kim, Eun-Nam, Jeong, Hye Gwang, Jeong and Gil-Saeng: Citropten Inhibits Vascular Smooth Muscle Cell Proliferation and Migration *via* the TRPV1 Receptor. ACS Omega 2024; 9: 29829-29839.
13. Mateus ARS, Teixeira JD, Barros SC, Almeida C, Silva S and Sanches-Silva A: Unlocking the potential of *Citrus medica* L.: antioxidant capacity and phenolic profile across peel, pulp, and seeds. Molecules 2024; 29(15):3533.
14. Zaric BL, Macvanin MT and Isenovic ER: Free radicals: Relationship to Human Diseases and Potential Therapeutic applications. Int J Biochem Cell Biol 2023; 154: 106346.
15. Entezari, Maliheh, Majd, Ahmad, Falahian, Fathollah, Mehrabian, Sedigheh, Hashemi, Mehrdad, Ardeshirylajimi and Abdolreza: Antimutagenicity and Anticancer Effects of Citrus Medica Fruit Juice. Acta Medica Iranica 2009; 47; 373-377.
16. Al-Yahya MA, Mothana RA, Al-Said MS, El-Tahir KE, Al-Sohaibani M and Rafatullah S: *Citrus medica* "Otroj": attenuates oxidative stress and cardiac dysrhythmia in isoproterenol-induced cardiomyopathy in rats. Nutrients 2013; 5(11): 4269-83.

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