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Extraction, Isolation and Phytochemical Investigation of Natural Products by Using Chromatographic (TLC) Method

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ABSTRACT

The use of natural products, especially plants, for healing is as ancient practice which is universally accepted. Natural products played a prominent role in ancient traditional medicine systems, such as Chinese, Ayurveda, and Egyptian, which are still in common use today. According to the World Health Organization (WHO), 75% of people still rely on plant-based traditional medicines for primary health care globally. The common problems and key challenges in the extraction, isolation and characterization of active ingredients in botanicals and herbal preparations are discussed routinely. The analysis of bioactive compounds present in the plant extracts involving the applications of common phytochemical screening assays, chromatographic techniques like HPLC and TLC. The current manuscript reports phytochemical investigation of the methanolic stem extract and its TLC analysis which showed the presence of glycosides, flavonoids, alkaloids, tannin compounds.

INTRODUCTION

Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity [1]. According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. The use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases [2]. Due to the development of adverse effects and microbial resistance to the chemically synthesized drugs, attention turned towards ethnopharmacognosy. They found literally thousands of phytochemicals from plants as safe and broadly effective alternatives with less adverse effect. Many beneficial biological activities such as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing activity were reported. In many cases the people claim the good benefit of certain natural or herbal products. However, clinical trials are necessary to demonstrate the effectiveness of a bioactive compound to verify this traditional claim. Clinical trials directed towards understanding the pharmacokinetics, bioavailability, efficacy, safety and drug interactions of newly developed bioactive compounds and their formulations (extracts) require a careful evaluation. Clinical trials are carefully planned to safeguard the health of the participants as well as answer specific research questions by evaluating for both immediate and long-term side effects and their outcomes are measured before the drug is widely applied to patients. According to the World Health Organization (WHO), nearly 20,000 medicinal plants exist in 91 countries including 12 mega biodiversity countries. The premier steps to utilize the biologically active compound from plant resources are extraction, pharmacological screening, isolation and characterization of bioactive compound, toxicological evaluation and clinical evaluation. A brief summary of the general approaches in extraction, isolation and characterization of bioactive compound from plants extract can be found in Figure 1. This paper provides details in extraction, isolation and characterization of bioactive compound from plants extract with common phytochemical screening assay, chromatographic techniques, such as HPLC, and HPLC/MS and Fourier Transform Mass Spectrometry (FTMS).

MATERIALS AND METHODS

Collection of plant material

The fresh Stem of *Azadirachta indica Linn (Neem)* was purchased from local nursery garden during the month of March 2016. The plant material was identified and authenticated at, Sri Krishna devaraya University. Botany Department by Dr. S. Thimma Naik. The fresh plant material was dried under shade. Dried plant material was powdered using mechanical grinder and passed through sieve no.60 to get the powder of desired coarseness. Powdered material was preserved in an air tight container.

Chemicals

Methanol, Diethyl ether, ethanol, $Fecl_{3,}$aluminum chloride, Ethyl acetate, *n*-Butanol,

Extraction

Dried the Stem of *Azadirachta indica Linn*(5 g) were extracted with 70% methanol (250ml) at refluxing temperature for 36 hrs. Aliquot of the obtained extract (20% v/v) was evaporated to dryness (methanol extract). The amount of 80% v/v of the extract was concentrated under the reduced pressure and the obtained product was fractioned using different organic solvents: petroleum ether, chloroform, ethyl acetate and *n*-butanol. The methanol,petroleum ether, chloroform, ethyl acetate, and the remaining water extracts were evaporated to dryness under reduced pressure. The yields of extracts were:

Methanol m = 0.2392 g Ethyl acetate m = 0.0984 g

Petroleum ether m = 0.0717 g*n*-Butanol m = 0.2391 g

Chloroform m = 0.0847 g Water m = 0.6819 g

Plant material

|

Successive Soxhlet extraction with different solvent

|

Preliminary fractionation by Chromatographic Technique

|

TLC/HPLC/VLC/CC/UV

Chromatographic techniques Thin-layer chromatography (TLC):

Separation by TLC is effected by the application of a mixture or extract as a spot or thin line onto a sorbent that has been applied to a backing plate. Analytical TLC plates (thickness 0.1–

0.2mm) are commercially available from suppliers such as Merck (e.g., the commonest analytical silica gel plate is the 20_20 cm, plastic or aluminum-backed Kieselgel 60 F254 plate having a 0.2mm thickness of silica sorbent [Merck No. 5554]). The plate is then placed into a tank with sufficient suitable solvent just to wet the lower edge of the plate/sorbent but not adequate to wet the part of the plate where the spots were applied (origin). The solvent front then migrates up the plate through the sorbent by capillary action, and this process is known as development(Fig. 1).

A factor in quantifying migration of a compound on a particular sorbent and solvent system is the Rf value. This is defined as

$$Rf = \frac{\text{Compound distance from origin midpoint}}{\text{Solvent front distance from origin}}$$

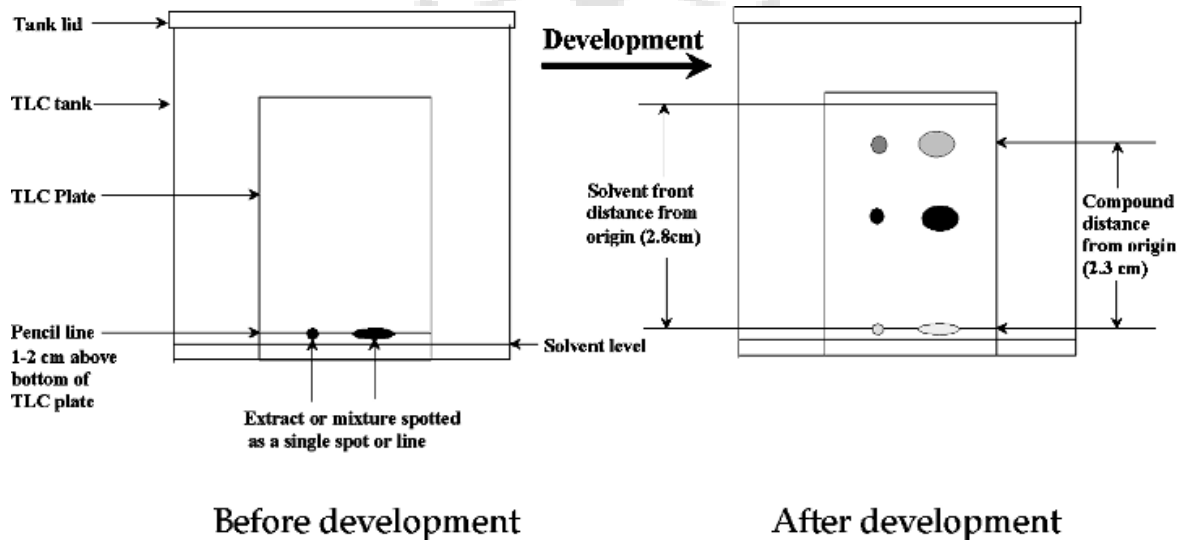


Fig:1

$$\frac{\text{Compound distance from origin 2:3 cm}}{\text{Solvent front distance from origin 2:8 cm}} = \text{Rf } 0.82$$

Rf values are always ratios, never greater than 1, and very depending on sorbent and/or solvent system. These values are sometimes quoted as hRf, i.e., relative to solvent front¼100, hRf¼Rf_100 (in our casehRf¼82).

Visualization/detection of compounds

Detection of compounds in TLC plates is a very important topic in analyzing extractives to isolate pure compounds. The following techniques are used for detecting the compounds in TLC/PTLC plates.

Visual detection

The developed chromatogram is viewed visually to detect the presence of colored compounds.

UV light

The developed and dried plates are observed under UV light of both long and short wavelength (254 nm and 366 nm) to detect the spot/band of any compound. Some of the compounds appear as fluorescent spots while the others as dark spots under UV light.

Iodine chamber

The developed chromatogram was placed in a closed chamber containing crystals of iodine and kept for few minutes. The compounds that appeared as brown spots are marked. Unsaturated compounds absorb iodine. Bound iodine was removed from the plate by air blowing.

Spray reagents

Different types of spray reagents are used depending upon the nature of compounds expected to be present in the fractions or the crude extracts.

a) Vanillin/H2SO4 [3]: 1% vanillin in concentrated sulfuric acid is used as a general spray reagent followed by heating the plates to 100^0C for 10 minutes.

b) Modified Dragendorff's reagent [4]:

Modified dragendorff's reagent was used to detect alkaloids. Some coumarins also give a positive test with modified dragendorff's reagent. The reagent is prepared by mixing equal parts (v/v) of 1.7 % bismuth subnitrate dissolved in 20 % acetic acid in water and a 40 % aqueous solution of potassium iodide.

c) Ferric chloride/EtOH [5]: Some of the phenolic compounds were detected by spraying the plates with ferric chloride (5% ferric chloride in absolute ethanol) reagent.

d) Perchloric acid reagent [6]: 2% aqueous perchloric acid produces brown spots with steroids after heating at 100^0C for 10 minutes.

e) Potassium permanganate reagent [7]: Only the oxidizable compounds were detected by this reagent. After spraying with the reagent the compound appeared as yellow or pale yellow spot on the colored (color of permanganate) plate.

Determination of Rf (retardation factor) values:

Rf value is characteristic of a compound in a specific solvent system. It helps in the identification of compounds. Rf value of a compound can be calculated by the
Following formula:

$$\text{Rf value} = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent system}}$$

Analysis of column fractions by TLC:

All the column fractions were screened by TLC under UV light and by spraying with vanillin sulphuric acid reagent. A number of compounds were detected, which were purified from the different sub-fractions employing various techniques. The isolated compounds have been summarized in**(Table 1)**.

Table 1: A list of isolated compounds from pet-ether and ethyl acetate, methanol, Ethanol soluble fractions

Sr. No.	Mobile phases	Rf value	isolated compound
1	Chloroform :Methanol acetate 12:2	0.8	Alkaloids
2	Ethyl acetate:Benzene	0.73	Terpenoids
3	Ethyl acetate:Butanol	0.87	Flavonoids
4	Methanol: water	0.83	Tannins

Qualitative analysis and thin layer chromatography Qualitative chemical analysis for alkaloids, tannins, flavonoids and terpenoids.

TLC Chromatogram of the investigated extracts

Fig: 2

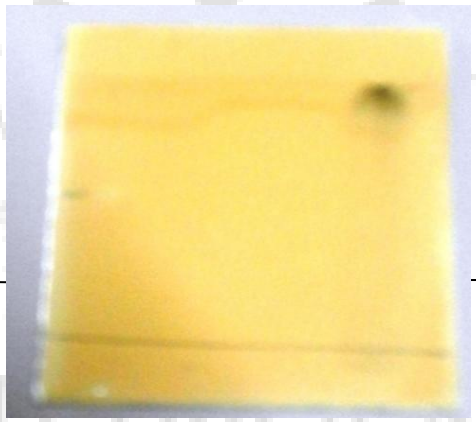
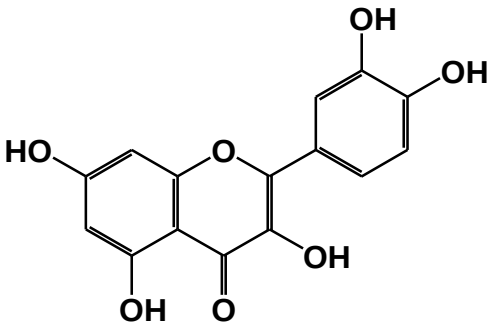


Fig: 3

FLAVONOIDS:

Flavonoids are low molecular weight [8-9] bioactive polyphenols[12] which play a vital role inphotosynthesizingcells[10]. The original "flavonoid" research apparently began in 1936, when Hungarian scientist Albert Szent-Gyorgi was uncovering a synergy between pure vitamin C and as yet unidentified co-factors from the peels of lemons, which he first called "citrin," and, later, "vitamin P" [11]. Flavonoids are secondary metabolites characterized by flavan nucleus [8] and C6-C8-C6 carbon-skeleton [12-13]. These are group of structurally related compounds with a chromane-type Skelton having phenyl substituent in C2-C3 position [15]. The basic structural feature of flavonoid is 2-phenyl-benzo-γ-pyrane nucleus

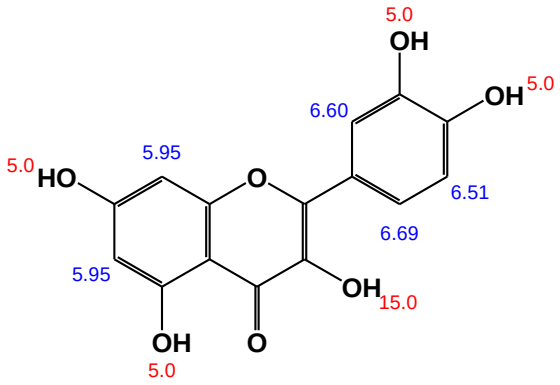
consisting of two benzene rings (A and B) linked through a heterocyclic pyran ring (C) as shown in fig (II) [10].



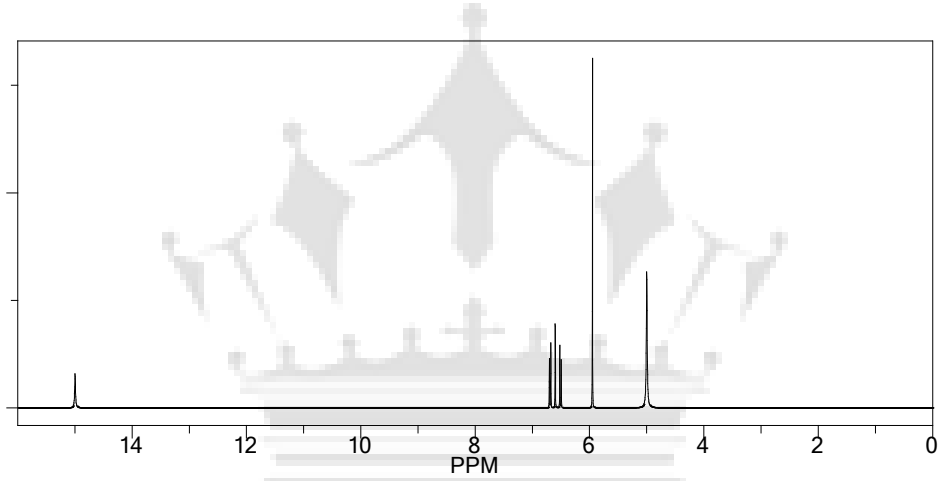
Quercetin

Fig-4

ChemNMR H-1 Estimation



Estimation Quality: blue = good, magenta = medium, red = rough



Protocol of the H-1 NMR Prediction:

Node	Shift	Base + Inc.	Comment (ppm rel. to TMS)
OH	15.0	15.00	enol
OH	5.0	5.00	aromatic C-OH
CH	5.95	7.26	1-benzene
		0.19	1 -C=O
		-0.53	1 -O
		-0.53	1 -O
		-0.44	1 -O
OH	5.0	5.00	aromatic C-OH
CH	5.95	7.26	1-benzene
		0.19	1 -C=O
		-0.44	1 -O
		-0.53	1 -O
		-0.53	1 -O
CH	6.60	7.26	1-benzene
		0.04	1 -C=C
		-0.53	1 -O
		-0.17	1 -O
OH	5.0	5.00	aromatic C-OH
OH	5.0	5.00	aromatic C-OH
CH	6.51	7.26	1-benzene
		-0.05	1 -C=C
		-0.17	1 -O
		-0.53	1 -O
CH	6.69	7.26	1-benzene
		0.04	1 -C=C
		-0.44	1 -O
		-0.17	1 -O

Alkaloids

Nimbin is a triterpenoid isolated from Neem. Nimbin is thought to be responsible for much of the biological activities of neem oil, and is reported to have anti-inflammatory, antipyretic, fungicidal, antihistamine and antiseptic properties.[14]

Alkaloids are a large group of nitrogen-containing secondary metabolites of plant, microbial or animal origin. The term originally implied pharmacologically active bases of plant origin, but the definition has subsequently been broadened so that it is now generally considered to include the majority of nitrogen containing natural products with the exception of the simple amino acids, proteins and nitrogen-containing substances of polyketide origin such as the aminoglycoside antibiotics. Basic properties may be weak or absent as in the various types of amide alkaloids. The class of microbial alkaloid overlaps

considerably with that of the nitrogenous antibiotics, and substances such as the cytochalasans which show antibiotic properties are in DNP classified as alkaloidal, the definition being a matter of semantics.Biogenetically and structurally the alkaloids are diverse and it is usual to discuss them in terms of biogenetic origin rather than purely on the basis of structural features.

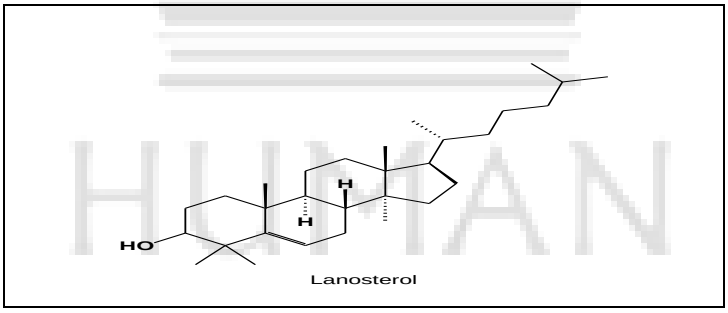


Fig -5Tannins

Plant polyphenols (vegetable tannins) are secondary metabolites widely distributed in the plant kingdom. Many reports on the use of SEC and GPC in the isolation of natural products are available in the literature. Most of them are used in conjunction with adsorption chromatography to isolate or purify compounds of interest, or to remove high molecular weight unwanted compounds, e.g., chlorophyll. However, the use of SEC is mainly restricted to the isolation and purification of proteins, peptides, and tannins. In the published protocols for the isolation of small natural compounds, Sephadex LH-20 is the

Most extensively used stationary phase. It has also been used routinely for the removal of chlorophyll from nonpolar or medium polarity natural product extracts.

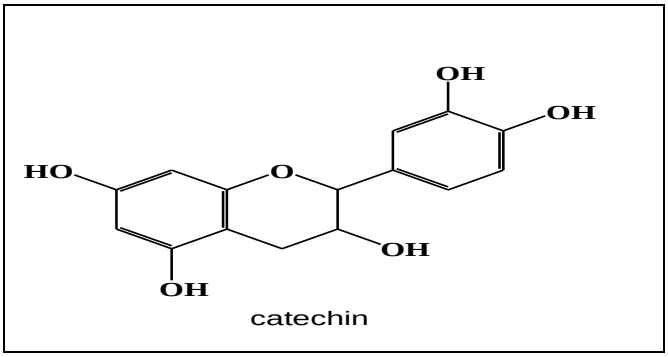
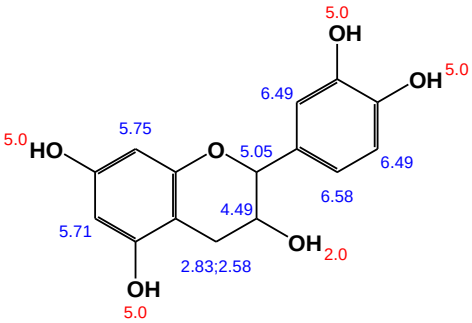
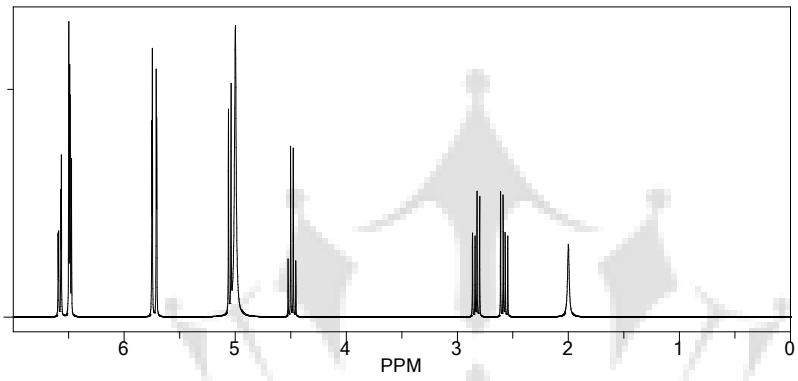


Fig-6

ChemNMR H-1 Estimation



Estimation Quality: blue = good, magenta = medium, red = rough



Protocol of the H-1 NMR Prediction:

Node	Shift	Base + Inc.	Comment (ppm rel. to TMS)
CH	5.05	1.50	methine
		1.28	1 alpha -1:C*C*C*C*C*C*1
		2.20	1 alpha -O-1:C*C*C*C*C*C*1
		0.08	1 beta -O
		-0.01	1 beta -C
CH	4.49	1.50	methine
		1.73	1 alpha -O
		0.38	1 beta -1:C*C*C*C*C*C*1
		0.50	1 beta -O-1:C*C*C*C*C*C*1
		0.38	1 beta -1:C*C*C*C*C*C*1
OH	2.0	2.00	alcohol
CH2	2.83;2.58	1.37	methylene
		1.22	1 alpha -1:C*C*C*C*C*C*1
		0.15	1 beta -O
		-0.04	1 beta -C
OH	5.0	5.00	aromatic C-OH
CH	5.71	7.26	1-benzene
		-0.05	1 -CC
		-0.53	1 -O
		-0.53	1 -O
		-0.44	1 -O-C
OH	5.0	5.00	aromatic C-OH
CH	5.75	7.26	1-benzene
		-0.05	1 -CC
		-0.44	1 -O
		-0.53	1 -O
		-0.49	1 -O-C
CH	6.49	7.26	1-benzene
		-0.07	1 -C-O
		-0.53	1 -O
		-0.17	1 -O
OH	5.0	5.00	aromatic C-OH
OH	5.0	5.00	aromatic C-OH
CH	6.49	7.26	1-benzene
		-0.07	1 -C-O
		-0.17	1 -O
		-0.53	1 -O
CH	6.58	7.26	1-benzene
		-0.07	1 -C-O
		-0.44	1 -O
		-0.17	1 -O

CONCLUSION

This study showed some important parameters to analyze bioactive compounds occurring in plant material, since bioactive compounds occurring in plant material consist of multi-component mixtures, their separation and determination still create problems. Practically most of them have to be purified by the combination of several chromatographic techniques and various other purification methods to isolate bioactive compound(s). Phytochemical investigation of Natural products by using Chromatographic (TLC) method, to determine the Flavonoid, Alkaloid, Tannins compounds.

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REFERENCES

1. Cosa, P., Vlietinck, A.J., Berghe, D.V., Maes, L. (2006). Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'. J. Ethnopharmacol. **106**: 290–302.
2. Duraipandiyan, V., Ayyanar, M. and Ignacimuthu, S. (2006). Antimicrobial activity of some ethnomedicinal plants used by Paliyar tribe from Tamil Nadu, India. BMC complementary Altern. Med. **6**: 35-41.
3. Stahl, E. (1969) 'Thin layer chromatography, A Laboratory Hand Book' (Revised and expanded 2nd edition). Springer, Verlog, Newyork.
4. Steinberg, D. M., Sokoll, L. J., Bowels, K. C., Nichols, J. H., Roberts, R., Schultheis, S. K. and O'Donnell, C. M. (1997) Clinical evaluation of Toxi-PrepTM: a semiautomated solid-phase extraction system for screening of drugs in urine. Clinical Chemistry, 43 : 2099-2105.
5. Lancashire, R. J. (1997) Qualitative analysis of organic compounds, The Department of Chemistry, University of the West Indies, Mona Campus, Kingston Jamaica.
6. Touchstone, J. C. and Dobbins, F. M. (1978) Practice of Thin Layer Chromatography, 1st Ed. John Willey and Sons.
7. Anal Chem resources; Analytical Chemistry resources (2006).
8. Heim, KE, Tagliaferro, AR, Bobliya, DJ. Flavonoids antioxidants: Chemistry, metabolism and structure-activity relationships. The Journal of Nutritional Biochemistry 2002; 13: 572-584.
9. Hollman, PCH, Katan, MB. Dietary Flavonoids: Intake, Health Effects and Bioavailability. Food and Chemical Toxicology 1999; 37: 937-942.
10. Cushnie, TPT, Lamb, AJ. Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents 2005; 26: 343-356.
11. Murray, MT. Quercetin: Nature's antihistamine. Better Nutrition 1998.
12. Peterson, J, Dwyer, MSJ, RD, DSc. Flavonoids: Dietary occurrence and biochemical activity. Nutrition Research 1998; 18: 1995-2018.

13. Tsuchiya, H. Structure-dependent membrane interaction of flavonoids associated with their bioactivity. Food Chemistry 2010; 120: 1089-1096.
14. Rijke, ED, Out, P, Niessen, WMA, Ariese, F, Goojer, C, Brinkman, UAT. 2006. Analytical separation and detection methods for flavonoids. Journal of Chromatography A 2006; 1112: 31-63.
15. W. Kraus, "Biologically active ingredients-azadirachtin and other triterpenoids", in: H. Schutterer (Ed.), The Neem Tree Azadirachta indica A. Juss and Other Meliaceous Plants, Weinheim, New York, 1995, p 35-88.