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Indexed in Elsevier Bibliographic Database (Scopus and EMBASE)
SCImago Journal Rank 0.129
Impact factor 0.67*

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Int J Pharm Bio Sci 2014 Jan; 5(1): (P) 606 - 611

Research Article **Pharmacognosy**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

ESTIMATION OF TOTAL PHENOLIC AND FLAVONOIDS CONTENT FROM EXTRACT OF *WRIGHTIA TINCTORIA* (ROXB) R. BR. LEAVES AND EVALUATION OF ITS ANTIOXIDANT ACTIVITY

T. SENTHIL KUMAR*[1], D. VENKAPPAYYA[1], VILAMBI N.R.K. REDDY[2] AND R. MANAVALAN[3].

[1]SASTRA University, Tirumalaisamudram, Thanjavur – 613401, India
[2]Trichy Pharma Chem P Ltd., VInYY Garden, Devadhanam. Trichy-620 002, India
[3]Institute of Pharmaceutical Technology, Annamalai University, Chidambaram, India

ABSTRACT

Flavonoids are widely distributed in Plant Kingdom that act as free radical scavengers and play a vital role in combating various diseases through their antioxidant property. An attempt was made to evaluate the antioxidant property of free and bound flavonoids present in *Wrightia tinctoria* (Roxb) R.Br. leaves (Apocynaceae). An initial estimation of total phenolic and flavonoid content after methanolic extraction of powdered leaves of *Wrightia tinctoria* (Roxb) R.Br. was done. Total phenolic content of 30.17 ± 2.36 mg/g (gallic acid equivalents /g of dry extract) and total flavonoid content of 21.33 ± 0.94 mg/g (quercetin equivalents /g dry extract) was obtained when estimated through Folin Ciocalteu reagent method and aluminum chloride colorimetric method respectively. The total antioxidant activity (by determination of phosphomolybdenum reduction) was found to be maximum [86.56 ± 2.20 mg/g (ascorbic acid equivalents /g of dry extract)] for free flavonoid fraction obtained from the methanolic extract when compared to the bound flavonoid extract (43.88 ± 2.56 mg/g). This result indicates that the polyphenols present in free flavonoid extract may be responsible for the enhanced activity. *Wrightia tinctoria* (Roxb) R.Br. is used for the treatment of skin infections since time immemorial and this finding on its antioxidant property documents a possible contributing mechanism by which it heals skin infections and provides an evidence for presence of polyphenols and free flavonoids in large quantity in its leaves.

KEYWORDS: Total phenolic content Total flavonoid content leaf extract *Wrightia tinctoria* (Roxb.) R. Antioxidant activity bound and free flavonoid

T. SENTHIL KUMAR
SASTRA University, Tirumalaisamudram, Thanjavur – 613401, India

***Corresponding author**

INTRODUCTION

There is a growing movement to find new medicines, or rediscover old ways of treating illness and improving general health. Flavonoids are phenolic substances widely distributed in the plants. They are a group of about 4000 naturally occurring compounds known to have contributed to human health through our daily diet[1]. They act in plants as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants, and for light screening. Many studies have suggested that flavonoids exhibit biological activities, including antiallergenic, antiviral, anti-inflammatory, and vasodilating actions. However, most interest has been devoted to the antioxidant activity of flavonoids, which is due to their ability to reduce free radical formation and to scavenge free radicals. The capacity of flavonoids to act as antioxidants *in vitro* has been the subject of several studies in the past years, and important structure−activity relationships of the antioxidant activity have been established. The antioxidant efficacy of flavonoids *in vivo* is less documented, presumably because of the limited knowledge on their uptake in humans. Most ingested flavonoids are extensively degraded to various phenolic acids, some of which still possess a radical-scavenging ability. Both the absorbed flavonoids and their metabolites may display an *in vivo* antioxidant activity, which is evidenced experimentally by the increase of the plasma antioxidant status, the sparing effect on vitamin E of erythrocyte membranes and low-density lipoproteins, and the preservation of erythrocyte membrane polyunsaturated fatty acids[2]. *Wrightia tinctoria* (Roxb.) R. Br. belongs to the family Apocynaceae. Its leaves were soaked in coconut oil for few hours and applied for eczema, psoriasis and other skin diseases[3]. In the present study methanolic extract was prepared from leaves of *Wrightia tinctoria* (Roxb) R.Br. which was further fractionated and screened for their antioxidant activity.

MATERIALS AND METHODS

1. Collection, authentication of plant material with chemicals used

The leaves of *Wrightia tinctoria* (Roxb) R.Br. were collected from VInYY garden, Nachallur, Karur district and identified by Prof. P. Jayaraman, of Plant Anatomy Research Centre (PARC), Chennai and a voucher specimen was deposited at PARC, Chennai. All chemicals and solvents were of analytical grade, gallic acid and ascorbic acid were obtained from Hi Media Laboratories Pvt. Ltd, Mumbai, India. Quercetin was obtained from Sigma Aldrich Chemical Co., USA. Double beam spectrophotometer (UV1240, Shimadzu, Japan), soxhlet extractor (Borosil), Balance (Dhonna), etc., were used during experimentation.

Extraction and fractionation of plant material

The leaves were collected and washed in running tap water to remove adhering dust materials. The cleaned leaves were shade-dried and finely powdered using blender for extraction. The dried and powdered leaves (100g) were soxhlet extracted[4] in 80 per cent methanol (500 mL) for 24 hours on a water bath. The extract was concentrated and divided into 2 portions. One portion was re-extracted with petroleum ether (fraction I), ethyl ether (fraction II) and ethyl acetate (fraction III) in succession. The petroleum ether extract was rejected as being rich in fatty substance. The ethyl ether fraction was analyzed for free flavonoids while the ethyl acetate fraction was hydrolyzed to cleave glycosides by refluxing with 7% H_2SO_4 for 2 hours. The resulting mixture was filtered and the filtrate was extracted with ethyl acetate in separating funnel. The ethyl acetate extract thus obtained was neutralized with 5% NaOH. The other portion of methanolic extract, ethyl ether fraction (free flavonoids) and ethyl acetate fraction (bound flavonoids) were dried *in vacuo* and weighed. The methanolic extract (1mg/mL) was used for the determination of total phenolic and total

flavonoid content. The methanolic extract, free and bound flavonoids were re-suspended in their respective solvents to get a concentration of 1 mg/mL (100 µg/0.1mL) and were used for testing antioxidant activity.

2. Total phenolic content

Total soluble phenolics in the extract were determined with Folin-Ciocalteu reagent using gallic acid (10-250 µg) as a standard phenolic compound[5]. 1.0 mL of extract solution containing 1.0 mg extract was diluted with 46 mL of distilled water in a volumetric flask. 1.0 mL of Folin-Ciocalteu reagent was added and the content of the flask mixed thoroughly. 3 minutes later 3.0 mL of 20% sodium carbonate was added and the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance of the blue color that developed was read at 760nm using a double beam spectrophotometer (UV1240, Shimadzu, Japan). The concentration of total phenols was expressed as gallic acid equivalents in mg/g of dry extract.

3. Total flavonoid content

Aluminum chloride colorimetric method was used for determination of flavonoids[6]. To the 10 mL volumetric flask 2 mL of water and 1 mL of plant extract (1 mg/mL) were added. After 5 minutes 3 mL of 5 % sodium nitrite and 0.3 mL of 10 % aluminum chloride were added. After 6 minutes, 2 mL of 1 M sodium hydroxide was added and the volume made up to 10 mL with water. Absorbance was measured at 510 nm. The total flavonoids were calculated from calibration curve of quercetin (10-250 µg) plotted by using the same procedure and was expressed as quercetin equivalents in milligrams per gram of dry extract.

4. Total antioxidant activity

Total antioxidant activity of the methanolic extract and its free and bound flavonoid fraction was evaluated by calculation through reduction of phosphomolybdenum according to the method of Prieto *et al*.,[7] an aliquot of 0.1 mL of sample (100 µg) solution was combined with 1mL of standard reagent (0.6 M Sulfuric acid, 28mM sodium molybdate and 4mM ammonium molybdate). The tubes containing the reaction mixture were capped and incubated in a boiling water bath at 95° C for 90 minutes. A typical blank solution contained 1 mL of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. Absorbance of all the samples was measured at 695nm. The antioxidant activity were calculated from the calibration curve of ascorbic acid (10-100 µg) plotted by using the same procedure and was expressed as ascorbic acid equivalents in milligrams per gram of dry extract.

STATISTICAL ANALYSIS

Three replicates of each sample were used for statistical analysis and the values are reported as mean ± standard deviation.

RESULTS AND DISCUSSION

Plants containing antioxidants like vitamin C, vitamin E, carotenes, polyphenols, flavonoids and many other compounds reduce disease risks against degenerative diseases[8]. Synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been used as additives; but for safety concerns, natural antioxidants are found to be the focus of intense interest. *Wrightia tinctoria* (Roxb) R.Br. leaf is observed to have been used in traditional practice for the treatment of skin infections. This initiated a curiosity to check its phenolic and flavonoid content with estimation of its antioxidant activity. Polyphenols scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and inhibit the oxidative mechanisms that can lead to degenerative diseases[9]. An initial estimation of total phenolic and flavonoid content after methanolic extraction of powdered leaves of *Wrightia tinctoria* (Roxb) R.Br. was done. The extraction protocol which has been carried out in the present investigation is exclusively meant for free and bound flavonoids. In the extraction procedure ethyl ether and ethyl acetate fractions are supposed to contain free and bound flavonoids, respectively. Here bound flavonoids imply that the flavonoids are

bounded with sugar moiety. These bounded sugar moieties are removed (from ethyl acetate fraction) by acid hydrolysis during extraction. If sugar part of the flavonoids is not removed, extracts do not react with spraying reagents (i.e. 5% fehling solution and 1% $AlCl_3$ solution) during TLC analysis. On the other hand, after removal of sugar part, flavonoids become free from sugars bounded to it and now react with spraying reagent and give colour reactions during TLC analysis. Spraying reagents 5% fehling solution and 1% $AlCl_3$ solution are exclusively used to detect flavonoids. Ethyl ether fraction contains free flavonoids i.e. flavonoids free from sugar moiety and during TLC analysis and thus shows positive colour reactions with the spraying reagents. In the ethyl ether fraction there is no need of acid hydrolysis as flavonoids in this fraction are not bound with sugar.

Total phenolic content of 30.17 ± 2.36 mg/g was obtained in methanolic extract of *Wrightia tinctoria* (Roxb) R.Br. when estimated through Folin Ciocalteu reagent method. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the Folin–Ciocalteu reagent [10] to form a blue complex that can be quantified by visible-light spectrophotometry[11]. The Folin-Ciocalteu method is described in several pharmacopoeias[12,13]. The reaction forms a blue chromophore constituted by a phosphotungsticphosphomolybdenum complex[11,14], where the maximum absorption of the chromophores depends on the alkaline solution and the concentration of phenolic compounds[11]. However, this reagent rapidly decomposes in alkaline solutions, which makes it necessary to use an enormous excess of the reagent to obtain a complete reaction. This excess can result in precipitates and high turbidity, making spectrophotometric analysis impossible. To solve this problem, Folin and Ciocalteu included lithium salts in the reagent, which prevented the turbidity[15]. The reaction generally provides accurate and specific data for several groups of phenolic compounds, because many compounds change color differently due to differences in unit mass[16]and reaction kinetics [15]. The results indicate that the total phenolic content in the methanolic extract of *Wrightia tinctoria* (Roxb) R.Br. is reasonably high. Flavonoids are one of the most diverse and widespread group of natural compounds and are probably the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties. A total flavonoid content of 21.33 ± 0.94 mg/g (quercetin equivalents / g dry extract) was obtained from the methanolic extract of *Wrightia tinctoria* (Roxb) R.Br. when estimated through aluminum chloride colorimetric method. The results are tabulated in Table No: 1

Table 1
Quantitative estimation of the total phenolic and total flavonoid contents in methanolic extract of Wrightia tinctoria (Roxb) R.Br. leaves

Wrightia tinctoria (Roxb) R.Br. leaves	total phenolic content (mg/g) ± S.D	total flavonoids content (mg/g) ± S.D
Methanolic extract	30.17 ± 2.36	21.33 ± 0.94

The basic principle to assess the antioxidant capacity through phosphomolybdenum assay includes the reduction of Mo (VI) to Mo (V) by the plant extract possessing antioxidant compounds. The total antioxidant capacity (by determination of phosphomolybdenum reduction) was found to be maximum [86.56 ± 2.20 mg/g (ascorbic acid equivalents /g of dry extract)] for free flavonoid fraction obtained from the methanolic extract when compared to the bound flavonoid extract (43.88 ± 2.56 mg/g). The methanolic extract when further fractionated, the petroleum ether extract fraction was rejected as being rich in fatty substance. The ethyl ether fraction was analyzed for free flavonoids[4] while the ethyl acetate fraction was hydrolyzed to cleave glycosides by refluxing with an acid. The resulting filtrate when extracted with ethyl acetate is observed to have bound

flavonoids[4]. Thus it is clear that the free flavonoid fraction contains polyphenolic compounds which are also evidenced through the high antioxidant activity value. The results are tabulated in Table No: 2.

Table 2
Total antioxidant activity of the various fractions of methanolic extract of Wrightia tinctoria (Roxb) R.Br. leaves

Wrightia tinctoria (Roxb) R.Br. leaves	total antioxidant activity in mg/g ± S.D
Methanolic extract	72.53 ± 1.36
Free flavonoids fraction	86.56 ± 2.20
Bound flavonoids fraction	43.88 ± 2.56

A vast amount of circumstantial evidence implicates oxygen derived free radicals such as superoxide and hydroxyl radicals as mediators of inflammation, shock and ischemial reperfusion injury [16]. Reactive oxygen species (ROS), initiate a wide range of toxic oxidation reactions and these toxicities are likely to play a prominent role in the pathophysiology of a number of diseases. There is a large amount of evidence to show that the production of ROS such as 0_2--, H_20_2, 'OH occurs at the site of inflammation and that they contribute to tissue damage [17]. Interventions which reduce the generation or effect of ROS exert beneficial effects in a variety of models of inflammation, shock and also in certain other pathophysiological conditions. In a number of disease conditions such as atherosclerosis, hypertension, ischaemic diseases, Alzheimer disease, Parkinsonism, cancer and inflammatory conditions imbalance between prooxidants and antioxidants is considered as a major cause. Phenolic compounds are among the major classes of antioxidants compounds. The phenolic compounds of plants fall into several categories such as simple phenolics, phenolic acids (derivatives of cinnamic and benzoic acids), coumarins, flavonoids, stilbenes, tannins and lignans[18].

Wrightia tinctoria (Roxb) R.Br. which is widely used for treatment of skin diseases is specifically used in the treatment of psoriasis. Psoriatic lesions are generally associated with histological changes like, Parakeratosis (presence of nucleated cells of the stratum corneum) Stratum granulosum (Its thickness decreases with increased disease) Spongiform pustule (degree of infiltration of polymorphs in the epidermis) Munro's microabscesss (infiltration of neutrophils in the epidermis) Acanthosis (elongation of reteridges) and Dermal vessel tortuosity (degree of tortuosity of dermal vessels). These histological changes have a great impact by the effect of ROS. Thus the present study has revealed that high total polyphenol content in the free flavonoids fraction of the methanolic extract increases the antioxidant activity and proves a linear correlation between phenolics content and antioxidant activity. So free flavonoids may be a contributing factor for the curative action of *Wrightia tinctoria* (Roxb) R.Br. leaf on the layers of skin.

CONCLUSION

These results suggest that there was a direct correlation between total phenolics and flavonoids content with antioxidant activity of leaves of *Wrightia tinctoria* (Roxb) R.Br. and they could contribute to the other bioactive characteristic of *Wrightia tinctoria* (Roxb) R.Br. as free radical oxidation is the main cause for many diseases.

ACKNOWLEDGEMENT

We are grateful to the Vice Chancellor and Research Dean of SASTRA University for their infrastructural facilitation and research motivation.

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