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IN VITRO PHYTOCHEMICAL STUDY ON BERBERIS ARISTATA ROOT EXTRACTS: AN EFFECTIVE NEUTRACEUTICAL FOR THE TREATMENT OF POLYCYSTIC OVARIAN SYNDROME (PCOS)

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ABSTRACT

Berberis aristata (tree turmeric) is widely known as "Daru haldi & Chitra" is spinous herb native to norther Himalyan region. Berberis aristata has been an integral part of ayurvedic medicines from long time with it's well known ant-inflammatory, anti-cancerous, anti-diabetic, anti bacterial potential. The research study aimed at evaluating the bioactive efficacy of medicinal plant under study by preparation of alcoholic and aqueous extracts of Berberis Aristata and comparing the bioactive content in each of the extracts. The study also included a microbial assay to determine the shelflife of the Berberis aqueous root extracts, longevity and duration of efficacy of the bioactives present in the food component extracts. The aqueous and alcoholic (methanolic) extracts were prepared for the comparative assessment of the presence of total phenol, total flavonoid and total tannin content. Total phenol and tannin content was determined using Folin-Ciocalteu's method and content was expressed as mg GAE(gallic acid equivalent) /g of the plant tissue while total flavonoid content was assessed by Aluminium calorimetric assay and it's amount was expressed as mg QE (quercetin equivalent)/g of the plant tissue. The major outcome expected out of the study included a higher total flavonoid content in both aqueous and alcoholic extracts of berberis as an extensive literature review confirms that higher intake of dietary flavonoids is inversely proportional to steroidogenesis which plays a major role in the pathophysiology of PCOS. Studies also revealed that higher the intake of total dietary flavonoids lower is the incidence of Type 2 Diabetes Mellitus and Metabolic syndrome in PCOS affected women.

INTRODUCTION

Polycystic Ovarian Syndrome commonly known as PCOS, is a very prevalent reproductive disorder in women and the leading cause of infertility among women today. The increasing trend of PCOS is predominantly seen in the age group of 15 to 30 years. An international consensus definition of PCOS has now been established which requires the fulfillment of atleast two of the following criteria: oligo and /or anovulation; clinical and/or biochemical signs of hyperandrogenism; and/or polycystic ovaries (Rotterdam Consensus, 2004).

Undiagnosed PCOS can lead to infertility and in long term can cause several health complications; which can be attributed to other factors as well. Researchers found that the incidence and prevalence of type-2 diabetes was three to five times higher in women with PCOS than in other women. In Karnataka, the prevalence of PCOS ranges from 11-26% (census-2013).

Due to the increasing prevalence of PCOS in India, there is a need for early diagnosis and treatment that can help relieve the symptoms and prevent health related problems. Several risk factors have been investigated in relation to PCOS, which includes obesity, glucose intolerance and dyslipidemia. Insulin resistance is known to play a critical role in the pathophysiology of PCOS. Many synthetic drugs exist for the effective treatment and management, however their numerous side effects and high cost have led a way to seek plant based remedies for the treatment of PCOS (Bency *et al.,* 2016).

Many medicinal plants have got significant activity in PCOS with fewer side effects accounting to their high antioxidant content. Apart from pharmacological therapy, lifestyle modification are equally significant in improving insulin sensitivity and hyperandrogenism. Therefore, these medicinal plant can be "paths or leads" to formulate newer synthetic compounds with greater therapeutic usefulness (Smitha *et al.,*

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2016). Medicinal plants are an important source of producing valuable bioactive secondary metabolites which are of significant importance in the health of individuals .The medicinal values of the plants are due to the chemical substances that produce a definite physiological action on human body. The antioxidants of the plant extracts have damaging effects on the oxidative free radicals that in turn play a major role in female reproductive system and female infertility (Lee *at al.,* 2010). Focus on the disease prevention by complementary supplementation of nutraceutical products to medication is now a growing demand for healthy food consumption. In addition, rising healthcare expenses have significantly boosted the growth of neutraceutical industry (Pandey *et al.,* 2013).

Neutraceuticals are food components or herbal extracts that have biological activity on the health of humans. They are products derived from food sources that are designed to provide extra health benefits, in addition to the basic nutritional value found in foods (Shahidi, 2009). One of many such novel medicinal plants include Berberis Aristata- having property of medicinally active bio-compounds helps in the treatment of ailments like type 2 DM, insulin resistance and PCOS. Metformin is commonly used drug/medicine in the treatment of Poly cystic ovarian disease and Type 2 Diabetes Mellitus.One of the natural sources of Metformin is Berberine i.e the active ingredient of Berberis Aristata is used in the form of extracts for product formulations due to their therapeutic effects on PCOS & T2 Diabetes Mellitus (Nestler, 2008).

The plant species used for the study is Berberis aristata (tree turmeric).It is widely known as "Daru haldi & Chitra" is spinous herb native to norther Himalyan region. Berberis aristata has been an integral part of ayurvedic medicines from long time. The plant alkoloid extracts were traditionally used in inflammation, wound healing, skin disease, diarrhoea, liver disorders, jaundice and many others (Komal *et al.,* 2011). A very valuable ayurvedic preparation "Rashut" is prepared by this plant which is an effective blood purifier. Hence, the plant fruit is edible & also rich source of vitamin C (Kurien *et al.,* 2007). The major bioactive found in B.Aristata root is berberine having yield of 2.23% followed by palmatine.

Berberine's main mechanism is partly responsible for its anti-diabetic and anti-inflammatory effects. Berberine is able to activate an enzyme called Adenosine Monophosphate-Activated Protein Kinase (AMPK) and inhibits the Protein-Tyrosine Phosphatase 1B (PTP1B). With the subsequent AMPK activation, glucose uptake into cells is doubled with improved insulin sensitivity, promoting regeneration and functional recovery of B-cells and reduction in the glucose production in the liver(Singh *et al*., 2010). The effect of AMPK activation is stimulation of hepatic fatty acid oxidation and ketogenesis, inhibition of cholesterol synthesis, lipogenesis, triglyceride synthesis, inhibition of adipocyte lipolysis, stimulation of skeletal muscle fatty acid oxidation, muscle glucose uptake and modulation of insulin secretion by pancreatic beta cells (Sabnis, 2006; Singhal and Sharma, 1976; Yanxia, 1995; Winder and Hardie, 1999).

Berberine has shown to regulate glucose and lipid metabolism invivo and invitro. A separate meta-analysis also revealed berberine has comparable therapeutic effect on type 2 DM [diabetes mellitus], hyperlipidemia and hypertension with no serious side effect with metformin. Treatment with berberine significantly decreased triglycerides, cholesterol, LDL, abnormal HbA1c levels and improved insulin receptor expression. Compared to metformin, berberine exhibited an identical effect in the regulation of glucose metabolism such as HbA1c, FBG, PBG, fasting insulin and post prandial insulin. This proves that berberine works as metformin for treating PCOS and insulin resistance. However in the regulation of lipid metabolism, berberine activity was better than metformin (Yin *et al.,* 2008; Dong *et al.,* 2012). Berberine has improved several clinical, metabolic & reproductive features in obese PCOS women & main effects could be related to the improvement of insulin sensitivity & reduction of hyperandrogenemia. It also seem to have greater effects on changes in body composition & dyslipidemia (Orio *et al.,* 2012).

Pharmacological studies conducted in India on the plant reveals its proven activity as an excellent hypoglycemic, antibacterial, antifungal, antipyretic, anti- inflammatory, hepatoprotective, antioxidant and anticancerous (Ranjan *et al.,* 2011). Each of these activities are attributed to their high antioxidants present like phenols and flavonoids in the root extracts. In comparison to other herbal extracts considered as GRAS (generally regarded as safe), berberine is a single purified compound and has glucose- lowering effect in vitro and in vivo. The extracts are proposed to decrease the HbA1c in comparison to metformin activity and also having potential as a therapeutic agent for lipid lowering. This activity is similar to that reported elsewere in vivo (Yin *et al.,* 2002).

MATERIALS AND METHODS

The root powder of Berberis Aristata was procured from Indian Herb Extraction, Nainital, India. The moist root of Berberis can also be obtained locally and sundried for three months.

Preparation of aqueous extracts

One gram of the root powder is weighed accurately and mixed homogenously in 100 ml of distilled water and converted into aqueous extracts.

The three aliquots chosen for the preparation of variants were:

30 ml
60ml
90 ml

Note: As mentioned before 1 g of each of the plant tissue was dissolved in 100 ml of warm water. This was done twice to obtain the first two portions of extract from first 100 ml aqueous solution and the third portion from the second 100 ml aqueous solution.

Preparation of alcoholic extracts

The collected plant samples were rinsed in distilled water, shade dried for 3 weeks and coarsely ground. The dry root powder obtained from the market can also be used for the analysis. Thereafter the root powder was macerated with motor and pestle and finely the macerated powder is used to prepare the extracts.

A total of 30g root powdered was weighed into a 500 ml conical flask and 300ml of methanol was poured into it. The mixture was shaken well, soaked for 72 hours with tight cotton plugging. The samples were centrifuged on the 3rd day at 4000rpm for 10-15 min. The procedure was repeated twice to collect all the supernatants into a 100ml round bottom flask. The extracts were filtered using Whatman No.1 filter paper and refrigerated immediately at 4°C.The extracts were used for the investigation of phytochemicals (Surya *et al.,* 2015; Ramamoorthy *et al.,* 2013)

Fig 1 Berberis root powder

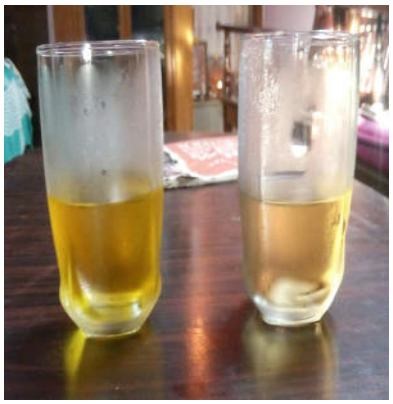


Fig 2 Aqueous extract

Fig 3 Initial methanolic extract

Fig4 Filtered methanolic extract

Fig 5 Refrigerated methanolic extract

Assessment of the presence and concentration of bioactive components in the prepared extracts

Qualitative analysis

The four groups of major bioactives that were analyzed in the Berberis extracts were: Phenols, Flavonoids, Tannins & Terpenoids. The extract of medicinal plants is analyzed for the presence of flavonoids, phenols, tannins and terpenoids according to standard methods (Edeoga *et al*., 2005;Yadav *et al*., 2012, Harborne *et al.,* 1973, Kokate *et al*., 2000) Both aqueous and methanolic extracts were used for screening the phytochemicals.

Screening for phenols

1ml of the extract was treated with 3% ferric chloride. If there is an appearance of deep blue color, then it shows the presence of phenol (Kokate, 2000; Harborne, 1973).

Screening for flavonoids

1ml of the extract was added with 1ml of sulphuric acid. Orange color formation confirmed the presence of flavonoids (Kokate, 2000; Harborne, 1973).

Screening for Tannins (Braymers Test)

1ml of the extract was added mixed with 2ml of water. To these 2 drops of 5% ferric chloride solution was added. Appearance of dirty green precipitate indicated the presence of tannins (Harborne, 1973; Edeoga, 2005).

Screening for terpenoids

2ml of the extract was added with 2ml acetic acid. Then concentrated sulphuric acid was added. Deep red color development showed the presence of terpenoids (Yadav, 2012).

Quantitative analysis

Quantitative estimation of bioactives namely total phenols, total flavonoids and total tannins in each of the plant extracts were performed using spectrophotometry. Spectrophotometry is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength .The equipment used for spectrophotometry is spectrophotometer -an apparatus for measuring the intensity of light in a part of the spectrum, especially as transmitted or emitted by particular substances.

O.D. is directly proportional to the concentration of the colored compound. Hence the unit of absorbance spectrophotometer is O.D. Most spectrophotometers have a scale that reads both in O.D. (absorbance) units, which is a logarithmic scale, and in % transmittance, which is an arithmetic scale.

Total phenolic content were determined using Folin-Ciocalteu's method

The procedure was performed referring to Singleton *et al.,* 1999 with minor modifications. Here gallic acid was used as the standard and the absorbance of the standard and test solutions were measured against the blank at 750nm. Calibration curve was plotted using standard gallic acid concentrations .The total phenolic content was expressed as mg of gallic acid equivalent weight(GAE)/g of extract.

Total tannin content was determined by Folin-Ciocalteu's method

The assessment was performed by referring to Rajeev *et al.*, 2005 with slight modifications. Here gallic acid was used as the standard. Absorbance of the test and standard solutions were measured against the blank at 725nm with UV/Visible spectrophotometer. The total tannin content was expressed in terms of mg of GAE/g of extract.

Total flavonoid content was measured with the aluminium chloride colorimetric/ spectrophotometric assay by referring to Lee & Safinar, 2012. Here absorbance of the standard and test solutions were measured at 510nm spectrophotometer. The calibration curve was plotted using standard quercetin. The total flavonoid content was expressed as mg of quercetin equivalents/g of extract.

Microbial analysis of the aqueous extracts

The aqueous extracts prepared were tested and analyzed in order to obtain the shelf life, longevity and duration of efficacy of the bioactives present in the food component extracts. The analysis performed constitutes:

Serial dilution(pour/spread plate)
Coliform forming units CFU) determination

The analysis was done in order to study the shelf life of the extracts and their bioactivity in-vitro.

The extracts were sealed and stored in plastic bottles and used during the testing. Serial dilution of each extract was done and then plating was performed using pour plate and spread plate method.

Nutrient Agar (NA) was used for pour plate method while Potato Dextrose Agar(PDA) was used for spread plate method. The dilutions were used in triplicates and the Colony Forming units (CFU) were counted.

Pour plate method was done using 1ml of the dilutions 10^{-7},10^{-8}, 10^{-9} and incubated at 35°C to 37°C for 24 hours. This is used to estimate the CFU for bacteria. Spread plate method was done using 0.1 ml of the dilutions 10^{-4}, 10^{-5}, 10^{-6} and incubated at room temperature (25 to 28°C) for 24-28 hours. This estimates the fungal count of the food.

Fig 6 Serial dilution

Fig 6 Plating

RESULTS

Qualitative phytochemical analysis:

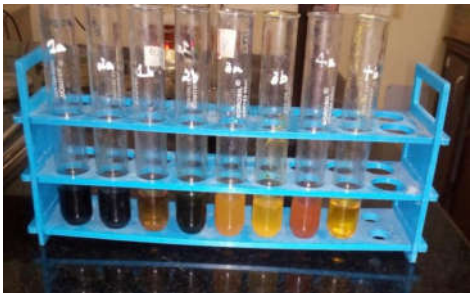


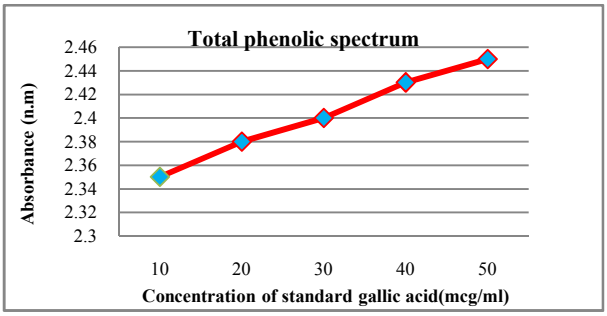
Fig 7 Phytochemical screening of the aqueous and methanolic extracts. Two tubes for each of the phytochemicals indicating first tube for aqueous and second for methanolic extracts. {phenols(left)-terpenoids(right)}

Table 1 Preliminary phytochemical screening of berberis extracts

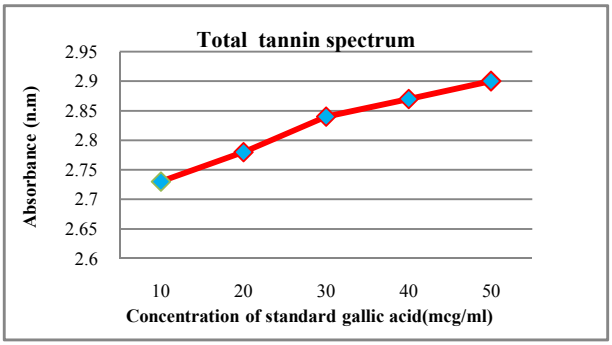
Experiment	Result	
	Aqueous Extract	Methanolic Extact
Test for phenols- 1 ml of extract is treated with 3% ferric chloride (few drops) Appearance of blue color shows the presence of phenols.	+	++
Test for tannins- I ml of extract is treated with 2ml of distilled water. Then treated with 2 drops of 5% ferric chloride. Appearance of dirty green precipitate indicates the presence of tannins.	+	+++
Test for flavonoids- 1 ml of extract is treated with 1ml sulphuric acid Orange color formation confirms the presence of flavonoids.	++	+++
Test for terpenoids- 2 ml of the extract is added to 2 ml acetic acid.Then concentrated sulphuric acid is added. Deep red color development showed the presence of terpenoids.	_	+

Note: + slightly present
++ moderately present
+++ significantly present
_ absent

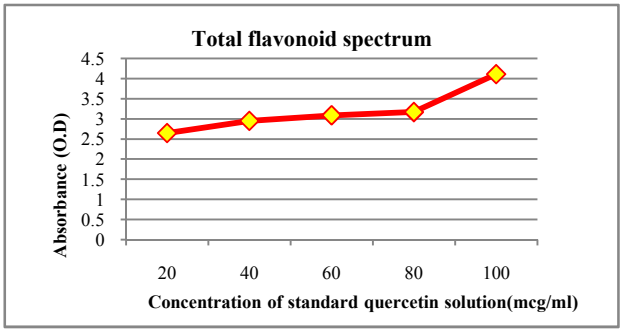
Quantitative phytochemical analysis



Graph 1 Standard calibration curve for gallic acid



Graph 2 Standard calibration curve for gallic acid



Graph 3 Standard calibration curve for quercetin solution

The key bioactives found in Berberis Aristata extracts were Berberine (protective alkaloid), total phenols(0.043 mg GAE/g), total tannins(0.056 mg GAE/ g) and total flavonoids(0.43 mg QE/ g).

The berberis root extract obtained from Indian Herb Extraction, Nainital was found to contain 98% pure berberine; which is a protective alkaloid having multiple therapeutic effects and targeting multifacet diseases like Type 2 DM, PCOS, insulin resistance, metabolic syndrome and cardiovascular diseases.

Table 2 Results of correlation

Phytochemicals	r	p	Significance
Phenol v/s tannin	0.301	0.105	Not significant
Phenol v/s flavonoid	0.369*	0.045	95%
Tannin v/s flavonoid	0.763**	0.000	99%

NOTE : ** Correlations are significant at 0.01 level
* Correlations are significant at 0.05 level
r = correlation
p= degree of significance

The results show the interdependence between the significant phytochemicals tested

Microbial assay

Table 3 Microbial analysis of berberine aqueous extracts

Product	Day	Appearance of bacterial and fungal colonies
Berberine aqueous extract	1	None
	7	None
	14	None
	21	None
	28	TLTC(Too low to count)

Microbial analysis state that the berberis extracts can be consumed safely till the 28 days of their preparation if incorporated into food products

DISCUSSION

Various studies on phytochemical screening reveal the presence of polyphenolic compounds in berebris aristata root extracts. In a study performed to detect the presence of phytochemical constituents in berberis root extracts, the alcoholic extracts(ethanolic+methanolic) showed significant presence of total tannins and total flavonoids and moderate presence of total phenols.While the aqueous showed slightly lower amounts of total phenols and total terpenoids while moderate amounts of total flavonoids and total tannins. This study further explained that all the protective properties of B.aristata is attributable to these phytoconstituents (Radhika and Vijaylakshmi, 2015).

According to a phytochemical study was conducted on berberis aristata stem and roots, the total flavonoid content and total polyphenol content were estimated using Aluminium Calorimetric Assay and Folin-Ciacolteu reagent 2 Assay at 510nm and 760nm respectively. The statistical analysis of the spectrophotometric results stated that berberis aristata contained 80.2±0.1 mg GAE/ g dw total polyphenols and 122±0.4 mg QE/ g dw of total flavonoids (Parajuli *et al*., 2012) According to a study analyzing the relationship between flavonoid intake and metabolic syndrome (MetS) in Korean women with PCOS states the significance of flavonoids in the treatment of MetS. The study selected 27 PCOS women with MetS and estimated their dietary intake using MDA (Mini

Dietary Assesment) score and intake of six flavonoid classes using a flavonoid data base.

The results stated that there was a significant inverse relationship between flavonol intake and risk of MetS, further concluding that higher the intake of flavonoids, lower is the risk of having PCOS related complications (Jisoo *et al.,* 2014).

Another study further states that possible mechanism of antioxidant activity is due to the presence of phytoconstituents like flavonoid and polyphenols present in the methanolic extracts of the plants. These plant parts can be used for isolating the active compounds and hence discovery of new drugs can be initiated. Total polyphenolic content (flavonoids and tannins) validated the idea behind the use of traditional medicinal plants to treat different diseases being possible sources of active compounds used for future study (Pokharel *et al*., 2015)

The correlation trend existing between total phenol, total tannin and total flavonoid content were observed simultaneously in the test plant extract. The total phenol and total tannin did not exhibit a significant positive correlation with $p>0.05$.This means as the total phenol content increased; the total tannin content did not increase in the plant extract.

However, a significant positive correlation was observed between total phenol and total flavonoid content. Therefore, it was concluded that as the total phenol content increased, there was a simultaneous increase in the total flavonoid content in the medicinal plant extracts with $p<0.05$.

While on the other hand; a higher positive correlation was observed between total tannin and total flavonoid content. This explained that as the total flavonoid content got elevated it led to a simultaneous rapid elevation in the total tannin content in both the plant extracts. These phytochemical contents had 99% significant correlation at $p <0.01$.

An ethnobotanical study on Berberis aristata DC. root extracts (methanolic and aqueous)show a wide antibacterial activity against gram-positive bacteria at a concentration of 50 µg/disc . Among the gram-negative bacteria tested, the antibacterial activity was limited against E. coli, Dysenteriae type 1 and the best activity being against V.cholerae The antibacterial activity of the extracts against clinical isolates was comparable to those of standard strains. It was also stated the methanolic and aqueous extracts of B.aristata showed best antimicrobial activity towards V.cholerae at 32.0±1.9 mcg/disc and 14.0±1.7mcg/disc (Shahid *et al.,* 2009).

CONCLUSION

The pure berberis extracts obtained in the study were found to be rich source of dietary total flavonoids as compared to other bioactive components studied. Therefore a major outcome of the study was accomplished, but further investigation of the extract properties need to be done for its effective incorporation into medicines and nutraceuticals. Dietary flavonoids have found to have close association with alleviating the symptoms of insulin resistance and PCOS. They not only propose a negative relationship with steroidogenesis but also improve insulin receptor functioning and further aid in weight loss.These plant extracts could be used for isolating an active compound and further be used for discovery of new drug in the future.

Recommendations of the Study

The methanolic and aqueous plant extracts were found to be rich sources of dietary total flavonoids which are PCOS protective Berberine extracts can be a potential drug for PCOS due to their significant metabolic correction and disease reversible effects..A randomized control trial (clinical intervention) could have been performed on PCOS women for 3 months to find out the efficacy of the health drinks which was not done due to time constraint. Further investigation of the bioactive efficiency should be conducted for the incorporation of extracts in a neutraceutical drug or capsule form.

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