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Standardization of Extraction Techniques of Picroside-I and Picroside-II from "Kutki" (*Picrorhiza kurroa* Royle *ex* Benth.)

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Abstract- Kutki (*Picrorhiza kurroa* Royle ex Benth.) is an important medicinal plant used in various herbal drug formulations possessing hepatoprotective activity. The pharmacological importance of this species has been demonstrated due to the presence of iridoid glycosides, such as picroside-I and picroside-II in underground part. In the present study, fresh roots and rhizomes collected from 2600m to 3300m altitude of Himachal Pradesh (Rohtang area) were used and extract yield, phytochemical constituents, namely picroside-I and picroside-II, of water and methanol extracts prepared using four different extraction methods viz. soxhlet, extraction by refluxing, microwave assisted extraction and sonication assisted extraction were compared for the standardization of extraction methods of picroside-I and picroside-II. Sonication assisted extraction for 36 minutes with methanol as solvent yielded 44.269 per cent extract with 6.825 per cent picroside-I and 5.291 per cent picroside-II content, which was better in comparison to other methods regarding time consumption and yield.

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Strictly as per the compliance and regulations of:

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Abstract- Kutki (*Picrorhiza kurroa* Royle ex Benth.) is an important medicinal plant used in various herbal drug formulations possessing hepatoprotective activity. The pharmacological importance of this species has been demonstrated due to the presence of iridoid glycosides, such as picroside-I and picroside-II in underground part. In the present study, fresh roots and rhizomes collected from 2600m to 3300m altitude of Himachal Pradesh (Rohtang area) were used and extract yield, phytochemical constituents, namely picroside-I and picroside-II, of water and methanol extracts prepared using four different extraction methods viz. soxhlet, extraction by refluxing, microwave assisted extraction and sonication assisted extraction were compared for the standardization of extraction methods of picroside-I and picroside-II. Sonication assisted extraction for 36 minutes with methanol as solvent yielded 44.269 per cent extract with 6.825 per cent picroside-I and 5.291 per cent picroside-II content, which was better in comparison to other methods regarding time consumption and yield.

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I. Introduction

Plants are an important source of bioactive molecules for drug discovery. Isolated bioactive molecules serve as starting materials for laboratory synthesis of drugs as well as a model for the production of biologically active compounds. Phytochemical processing of raw plant materials is essentially required to optimize the concentration of known constituents and also to maintain their activities (Aziz *et al*., 2003). Extraction is an important step in the itinerary of phytochemical processing for the discovery of bioactive constituents from plant materials.

The general techniques of medicinal plant extraction include maceration, infusion, percolation, digestion, decoction, hot continuous extraction(Soxhlet), aqueous-alcoholic extraction by fermentation, counter-current extraction, microwave assisted solvent extraction, ultrasound assisted solvent extraction (sonication), supercritical fluid extraction and phytonic extraction (Handa *et al*., 2008; Co *et al*., 2012). Selection of a suitable extraction technique is important for the standardization of herbal products as it is utilized in the removal of desirable soluble constituents, leaving out those not required with the aid of the solvents.

Use of green extraction techniques such as Ultrasound Assisted Solvent Extraction (UASE) (Wu *et al*., 2001; Ahh *et al*., 2007), Microwave Assisted Solvent Extraction(MASE) (Ganzler *etal*., 1986a,b) and Supercritical Fluid Extraction (SFE) (Ollanketo *et al*., 2002) has been rapidly and continuously increasing globally for phytochemical processing of medicinal plants as these techniques are fast as compared to traditional methods. Also, these techniques are environmentally friendly regarding solvent and energy consumption. Yield is also comparable to conventional extraction and in some cases it is even higher. However, extract yield as well as the bioactivities of the extract prepared using different extraction methods have been reported to vary in several studies (Hayouni *et al*., 2007).

The drug "Kutki" consists of the dried rhizomes and roots of *Picrorhiza kurroa* Royle *ex* Benth., which is an important alpine herb of Himalayan region growing at an altitudinal range of 3,000 to 5000 m above mean sea level (Kaul and Kaul, 1996; Anonymous, 2001; Vinoth *et al*., 2010). It is endemic to Western Himalayas extending up to mountains of Yunnan in China (Anonymous, 1969).

It is a well-known drug in the Ayurvedic system of medicine and extensively used in traditional system of medicine in India, China, Tibet, Nepal and Sri Lanka for the treatment of various immune-related diseases (Bhandari *et al*., 2008). The medicinal importance of *Picrorhiza kurroa* is due to its pharmacological properties like hepatoprotective (Chander *et al*., 1992), antioxidant (Ansari *et al*., 1998), antiallergic and antiasthmatic (Dorch *et al*., 1991), anticancerous activity particularly in liver (Joy *et al*., 2000), and immunomodulatory, (Gupta *et al*., 2006).

More than 50 secondary metabolites have been reported from the plant *Picrorhiza kurroa* which includes iridoid glycosides, cucurbitacins and phenolic compounds. The pharmacological importance of *Picrorhiza kurroa* has been demonstrated due to the presence of iridoid glycosides, such as picroside-I and picroside-II (Rastogi *et al*., 1996).

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With increasing demand for herbal medicinal plants, and natural products for health care all over the world, herbal manufacturers aim at using the most appropriate extraction technologies to produce extracts of defined quality and quantity with least batch to batch variation. Standardization of extraction procedures contributes significantly to the final quality and quantity of the product. The selection of method to isolate active components with the best yield and high purity with minimum inputs is mainly dependent on the nature of compounds and raw material which to be processed (Kothari *et al*., 2009).

The basic parameters influencing the quality of an extract are plant parts used as starting material, the solvent used for extraction and extraction procedure (Ncube *et al*., 2008).

Keeping in view to find the correlation between different extraction methods, extraction yield, total phyto constituents picroside-I and picroside-II and to standardize the extraction techniques, the present investigation was carried out.

II. Materials and Methods

a) Plant material and extract preparation

Fresh roots and rhizomes of *Picrorhiza kurroa* were collected from 2600m to 3300m altitude of Himachal Pradesh (Rohtang area) in 2012 and harvested material was washed with water to remove the soil and other adhering material and then cut into small pieces and dried under shade. The air-dried material was then ground to form uniform powdered material. Accurately weighed air-dried and powdered plant material (2g) each was subjected to extract using five extraction techniques, namely soxhlet, reflux, sonication, microwave with methanol and methanol: water (90:10).

For soxhlet extraction, thimble having 2g powdered plant material prepared and extracted with methanol for 4 hours, 8 hours, 12 hours, 16 hours, 20 hours and 24 hours separately at 100° C on water bath. After extraction, the solvent was distilled off and the residue was dried to a constant weight and yield of extract was recorded.

For refluxing, 2g of powdered plant material was mixed with methanol in a round bottom flask and refluxed for 2 hours, 4 hours, 6 hours, 8 hours 10 hours and 12 hours separately at 100° C on water bath. Liquid extracts obtained were separated from the solid residue by vacuum filtration and the solvent was distilled off and dried to a constant weight and yield of extract was recorded.

For ultrasound assisted sonication extraction, air dried powdered plant material (2g) was mixed with methanol in a flask and extracted for 20 minutes, 24 minutes, 28 minutes, 32 minutes, 36 minutes and 40 minutes separately. Temperature was maintained at 35°C ± 1°C and sonication power was 120 MHz. After extraction, the material was filtered and then solvent from the filtrate was distilled off and the residue was dried to a constant weight and yield of extract was recorded.

Similarly, for microwave assisted solvent extraction, powdered plant material (2g) was mixed with methanol and methanol: water (90:10) separately in the flask and extracted for 20 minutes, 24 minutes, 28 minutes, 32 minutes, 36 minutes and 40 minutes separately. The domestic microwave oven of IFB brand model 30SC3 was used for microwave assisted extraction. Microwave output power was maintained 180 watts (20 % out of 900 watts). The supernatant was similarly preceded as described in UASE to get dried MASE extract of *Picrorhiza kurroa*.

The yield of the extract obtained from the different extraction methods was calculated by using the following formula.

$$\text{Yield (\%)} = \frac{\text{The weight of the residue obtained}}{\text{The weight of the plant material taken}} \times 100$$

b) Quantitative estimation of picroside-I and picroside-II in extracts using high-performance liquid chromatography (HPLC)

Picroside-I and picroside-II were quantified in the extracts using HPLC (Water's binary HPLC unit with Waters HPLC pump 515and dual λ absorbance detector 2487) on column Sunfire C-18 (4.6 × 250 mm, 5μm). The mobile phase consisted of methanol: water (40: 60) with the flow rate of 0.9 ml/min. The temperature was maintained $24^0 \pm 1^0$ C.

Each dried extract was 1000 times diluted with mobile phase, filtered through the membrane filter and 20μl of each sample was injected in HPLC, and Area Under Curve for peaks of picroside-I and picroside-II was recorded at 270 nm wavelength on the basis of retention time with the comparison of the retention time of standard picroside-I and picroside-II, their contents in the extracts were quantified on the basis of area of peak.

Percentage of Picroside-I and picroside-II in each sample was calculated by using the following formula where percent purity of the standard compound was taken as 95%:

$$\text{Picrosides content(\%)} = \frac{\text{Test Area}}{\text{Standard Area}} \times \frac{\text{Wt. of Standard}}{\text{Standard Dilution}} \times \frac{\text{Test Dilution}}{\text{Test Weight}} \times 100 \times \text{Percent Purity}$$

III. Results and Discussion

Biologically active compounds usually occur in low concentration in plants. An extraction technique is that which can obtain extracts with high yield and with minimal changes to the functional properties of the extract required (Quispe Candori *et al*., 2008). Several studies have reported variations in the biological activities of extracts prepared using different extraction techniques. Therefore, it is necessary to select the suitable extraction method as well as solvent based on sample matrix properties, chemical properties of the analytes, matrix-analyte interaction, efficiency and desired properties (Hayouni *et al*., 2007; Ishida *et al*., 2001).

In conventional extraction, heat is transferred through convection and conduction from the surface, here, the extractability of solvents depends mainly on the solubility of the compound in the solvent, the mass transfer kinetics of the product and the strength of solute/matrix interaction with corresponding limitations on heat and mass diffusion rate. Ultrasound assisted solvent extraction is a process that uses high intensity, high frequency sound wave sand solvents to extract targeted compounds from various matrices. Physical and chemical properties of the materials subjected to ultrasound are altered due to the propagation and interaction of sound waves as they disrupt the plant cell walls, thereby, facilitating the release of extractable compounds and enhancing mass transport of solvent from the continuous phase into plant cells. Microwave energy and solvents are used for the extraction of targeted compounds from plant matrices in the case of microwave assisted solvent extraction. Highly localized temperature and pressure can cause selective migration of targeted compounds from the matrices to the surroundings at a more rapid rate. Recoveries are similar or better in both UASE and MASE as compared to conventional extraction. However, reduced extraction time and solvent consumption are the main advantages of UASE and MASE. Although, extraction of bioactive compounds from the plants has been extensively investigated using conventional solvent extraction, the present investigation was undertaken to study the effect of extraction duration on phytochemical extraction through different methods on yield and phytochemical qualities of *P. kurroa* and after standardization of individual method, all the methods were compared so as to find out the best extraction method with maximum picroside-I and picroside-II content.

Extract yield from roots and rhizomes of *P. kurroa* prepared by soxhlet, refluxing, UASE and MASE methods under different duration using methanol and methanol-water, and the present quantity of phytochemical constituents (picroside-I and picroside-II) is summarized in Table 1 to 5 and the comparison among different methods of extraction is presented in Table 6.

In soxhlet extraction, 4 hours, 8 hours, 12 hours, 16 hours, 20 hours and 24 hours were used for the extraction of raw material, where extraction up to 12 hours was found the best time of extraction with maximum extraction yield (28.931%), picroside-I (5.937%) and picroside-II (5.212%).

In reflux extraction method, 2 hours, 4 hours, 6 hours, 8 hours, 10 hours and 12 hours were used for the extraction of raw materials, where 6 hours was found the best time of extraction with maximum extraction yield (22.713%), picroside-I (5.991%) and picroside-II (5.120%).

In sonication method, 20 minutes, 24 minutes, 28 minutes, 32 minutes, 36 minutes and 40 minutes were used for the extraction of raw materials, where 36 minutes was the best time of extraction with maximum extraction yield (44.269%), picroside-I (6.825%) and picroside-II (5.291%).

Similarly, in microwave, 100% methanol and methanol: water (90:10) method, similar time periods (20 minutes, 24 minutes, 28 minutes, 32 minutes, 36 minutes and 40 minutes) were used to extract raw materials, where 28 minutes was found the best time of extraction with maximum extract yield and phytochemical content for both methods , however the quantity of extract yield and phytoconstituents varied. In former, extract yield recorded (23.488%), picroside-I (2.642%) and picroside-II (2.192%) and in later, extract yield (15.346%), picroside-I (1.878%) and picroside-II (1.489%) was recorded. It showed improvement of yield and bioactive content in case of the method where solvent was only methanol instead of the mixture of methanol and water.

The comparison carried out among different extraction methods after standardization of extraction duration under different extraction methods showed sonication assisted extraction method to be the best method for 36 minutes extraction with maximum total extract (44.269%), maximum picroside-I content (6.825%) and maximum picroside-II content (5.291%).

The similar results have been reported by Sun *et al.,* (2011), Jadhav *et al.,* (2009), Xia *et al.,* (2006), Smelcerovic *et al.,* (2006), and Bimakr *et al.,* (2013) while comparison was carried out among various extraction methods for the various phytoconstituents from various plant materials.

Table 1: Effect of Extraction Duration on Phytochemical Extraction through Soxhlet Extraction Method

Extraction duration	Total extract%	Picroside-I (%)	Picroside-II (%)
4 hours	24.225	5.515 (2.552)	4.774 (2.403)
8 hours	25.486	5.833 (2.614)	4.850 (2.418)
12 hours	28.913	5.975 (2.641)	5.212 (2.492)
16 hours	29.425	6.052 (2.655)	5.227 (2.495)
20 hours	29.988	6.062 (2.657)	5.228 (2.496)
24 hours	30.000	6.062 (2.657)	5.223 (2.494)

(Values in parentheses are transformed values using $\sqrt{x+1}$ transformed values)

Table 2: Effect of Extraction Duration on Phytochemical Extraction through Reflux Extraction Method

Extraction duration	Total extract (%)	Picroside-I (%)	Picroside-II (%)
2 hours	6.200	5.663 (2.581)	4.481 (2.314)
4 hours	8.175	5.805 (2.609)	4.538 (2.353)
6 hours	22.713	5.991 (2.644)	5.120 (2.474)
8 hours	32.688	6.011 (2.648)	5.125 (2.475)
10 hours	34.050	5.945 (2.635)	5.124 (2.474)
12 hours	34.988	5.923 (2.631)	5.116 (2.473)

(Values in parentheses are transformed values using $\sqrt{x+1}$ transformed values)

Table 3: Effect of Extraction Duration on Phytochemical Extraction through Sonication Extraction Method

Extraction duration	Total extract (%)	Picroside-I (%)	Picroside-II (%)
20 minutes	33.013	5.826 (2.610)	4.702 (2.387)
24 minutes	38.018	6.459 (2.732)	5.011 (2.452)
28 minutes	40.994	6.621 (2.760)	5.235 (2.497)
32 minutes	42.533	6.799 (2.792)	5.286 (2.507)
36 minutes	44.269	6.825 (2.797)	5.291 (2.508)
40 minutes	45.135	6.825 (2.797)	5.293 (2.508)

(Values in parentheses are transformed values using $\sqrt{x+1}$ transformed values)

Table 4: Effect of Extraction Duration on Phytochemical Extraction through microwave, Methanol: Water (90:10) Extraction Method

Extraction duration	Total extract(%)	Picroside-I (%)	Picroside-I (%)
20 min	4.668	1.509(1.584)	1.285(1.512)
24 min	5.584	1.685(1.639)	1.398(1.548)
28 min	15.346	1.878(1.696)	1.489(1.578)
32 min	17.495	1.853(1.689)	1.433(1.560)
36 min	21.553	1.802(1.674)	1.398(1.548)
40 min	25.54	1.776(1.666)	1.361(1.537)

(Values in parentheses are transformed values using $\sqrt{x+1}$ transformed values)

Table 5: Effect of Extraction Duration on Phytochemical Extraction through Microwave, Methanol 100% Extraction Method

Extraction duration	Total extract (%)	Picroside-I (%)	Picroside-II (%)
20 minutes	5.916	1.735 (1.654)	1.435 (1.560)
24 minutes	9.306	2.613 (1.901)	1.993 (1.730)
28 minutes	23.488	2.642 (1.908)	2.192 (1.786)
32 minutes	24.742	2.554 (1.885)	2.045 (1.745)
36 minutes	30.316	2.478 (1.865)	1.977 (1.725)
40 minutes	34.425	2.415 (1.848)	1.798 (1.672)

(Values in parentheses are transformed values using $\sqrt{x+1}$ transformed values)

Table 6: Effect of Different Extraction Methods on Total Extract, Picroside-I and Picroside- II Content (%)

Extraction method	Extraction time	Total extract %	Picroside-I %	Picroside-II%
Soxhlet extraction	12 hours	28.913	5.975 (2.641)	5.212 (2.492)
Reflux extraction	6 hours	22.713	5.991 (2.644)	5.120 (2.474)
Sonication	36 minutes	44.269	6.825 (2.797)	5.291 (2.508)
Microwave, methanol: water (90:10)	28 minutes	15.346	1.878 (1.696)	1.489 (1.578)
Microwave,100% methanol	28 minutes	23.488	2.642 (1.908)	2.192 (1.786)

(Values in parentheses are transformed values using $\sqrt{x+1}$transformed values)

IV. Conclusion

The standardization of the extraction process is very important for the isolation of bioactive compound. In the present work, the results indicate that ultrasound assisted sonication extraction method can be viable alternative method to extract higher yield along with phytoconstituents (picroside-I and picroside-II). The result also reveals that the methanol can be used as a suitable solvent rather than the mixture of methanol-water for extraction of polar compounds.

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