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Comparative phytochemical analysis on ethanolic extract obtained by soxhlet and microwave extraction of *Canscora decurrens* Dalz.

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Abstract
Wide range of technologies are available for the extraction of active components from medicinal plants. Microwaves are used for extraction of phytoconstituents from plant material because of impressive research interest and potential. Conventional extraction techniques are time consuming and require more solvent and most of them are not suitable for thermolabile constituents. The microwave extraction techniques were found to be better than conventional methods as they require less time, consume less solvent. Significant features of the present study is that the microwave extraction method was successful in extracting various phytoconstituent. The two methods of extraction for phytoconstituent are compared in terms of no. of bands generated on TLC by individual phytochemical groups.

Keywords: *Canscora decurrens*, *Gentianaceae*, microwave, soxhlet, phytochemical groups, phytochemistry

1. Introduction
Genus *Canscora decurrens* (2n=38) (Syn. of *C. diffusa*) belongs to family *Gentianaceae*, which includes 99 genera and approximately 1,736 species. Some of them like, Swertia, Gentiana, Centaurium, and Canscora are routinely used in traditional medicines [37]. This genus is included in the famous΄Shankhpushpi' group of plants including *Convolvulus microphyllus*, *Evolvulus alsinoides* and *Clitoria ternatea* which are used as ingredient in formulation used to improve intelligence, memory and other higher mental function [6, 25]. *Canscora decurrens* (syn. *C. diffusa*) is such a potential medicinal plant known to cure large number of disorders of central nervous system. Although it's medicinal uses are well documented in old literature [9]. The medicinal properties in plants are useful for healing as well as for curing of human diseases because of the presence of phytochemical constituents [23]. Phytochemicals are naturally occurring in the medicinal plants, leaves, vegetables and roots that have defense mechanism and protect from various diseases. Phytochemicals are primary and secondary compounds. Chlorophyll, proteins and common sugars are included in primary constituents and secondary compounds have terpenoid, alkaloids and phenolic compounds [29]. These phytochemicals form a natural defense system for host plant and provide aroma, colour and flavor. Therapeutic properties of any medicinal plant can be attributed to these phytochemicals. More than 4,000 phytochemicals have been discovered in this respect [18]. Success of phytochemical analysis largely depends on the methods of extraction, resolution of phytoconstituents and detection. The history of plants used for medicinal purpose is probably as old as the history of mankind. Extraction is one of the critical steps in achieving complete recovery of desired phytoconstituent. For that, development of high performance and rapid extraction method is imperative. Traditionally maceration, percolation, sonication and soxhlet extraction methods are used. But all of them are time consuming and require large quantities of organic solvents. Search for new extraction techniques with shorter time and requiring very less amounts of solvents led to modern extraction methods like microwave assisted extraction(MAE),Supercritical fluid extraction (SCFE) and others. Microwaves are electromagnetic radiations with frequency from 0.3 to 300 GHz. In order to avoid interferences with radio communications, domestic and industrial microwaves generally operate at 2.45GHz [2]. Microwaves are made up of two oscillating perpendicular field's i.e. electric field and magnetic field and the former is responsible for heating. Unlike conventional heating which depends on conduction–convection phenomenon with eventually much of the heat energy being lost to the environment, in case of MAE, heating occurs in a targeted and selective manner with practically no heat being lost to the environment as the heating occurs in a closed system.

This unique heating mechanism can significantly reduce the extraction time (usually less than 30 min) as compared to Soxhlet [26, 30]. The present paper deal with the two methods of extraction i, e microwave and soxhlet on ethanolic extract of *C. decurrens* and phytoconstituent are compared in terms of no. of bands as well as phytochemical groups and preliminary separation and detection of phytochemical compounds was subjected to TLC technique.

2. Materials and Method

(1) Extraction: To compare phytochemical output, two types of extraction methods were used

(1) By soxhlet extractor for 12hrs.

(2) By microwave oven for 4 minutes.

- **Soxhlet extraction:** 5 gram powder was taken and extracted with 150 ml of ethanol at (60 ºC) for 12 hrs. The extract was filtered with whattmanno.1 filter paper and evaporated at room temperature and residue was weighed and dissolved suitably in alcohol so that stock of 0.5gm\ml concentration was obtained. It was stored in amber coloured bottle in refrigerator for detection of different phytochemical groups.
- **Microwave extraction:** 500 mg powder was taken in and extracted 15 ml ethanol, mixed well and placed in the water bath in microwave (Kenstar3D power and set at 60%) in 8 cycles of 30sec for 4 min [21].The extract was filtered with cotton and evaporated at room temperature. The residue was weighed, stored in labeled amber coloured bottles in the refrigerator and used for the detection of different phytochemical groups.

(2) Separation

- **Thin layer chromatography (TLC) plate preparation:** 20g of silica gel G (Merck) was mixed with 40ml distilled water to make a slurry. The slurry was then poured in an applicator to cast on cleaned TLC plates of the dimension 12 x 22cm. The plates were dried and activated by keeping them in oven at 60 ºC overnight. The plates were used for further analysis.
- **Application of sample:** Residues obtained from soxhlet and microwave extraction were weighed and a stock was made and loaded on TLC plate and left for drying. The plates were developed in developing chamber using different mobile phases up to 15cm. Plates were run in duplicate. Protocols were standardized for qualitative detection of alkaloids, anthracene, coumarins, flavonoids, phenolic acids tripenoids, xanthones, bitter principle.

(3) Identification

The separated bands were visualized by using different derivatizing reagents. The plates were observed in UV254, UV 365 and visible light before and after chemical treatment (table1).

Different phytochemical groups were identified by colour, no. of bands, intensity and Rf value of band. Rf value is calculated by following formula:

Rf = Distance travelled by the solute/ distance travelled by the solvent.

3. Results

Both the types of extract indicated presence of phenolic compounds (phenol acid, anthracene, bitter principle, coumarins, flavonoids, Xanthones (fig.1, table.1).

Table 1: Comparative phytochemical analysis of ethanolic extract of *C. decurrens.*

Compounds	Solvent system	Spray	Colour of band	No. of Band		Rf value	
				Soxhlet	Microwave	Soxhlet	Microwave
Phenolic compounds	Ethylacetate: benzene(9:11)	Folin-ciocaultu	Bluish green in visible	7	7	0.27,0.39,0.60,0.68,0.74, 0.90,0.96	0.25,0.36,0.59,0.64,0.71,0.92,0.97
Phenolic acids	Ethylacetate: methanol: water(100:13.5:10)	10%Ethanolic KOH	fluorescent bluish white band in uv 365nm	6	7	0.43,0.51,0.58,0.67,0.84, 0.90	0.41,0.48,0.52,0.56,0.62,0.86,0.91
Anthracene	Ethylacetate: methanol: water(100:13.5:10)	10%Ethanolic KOH	yollow band in visible	4	4	0.43,0.52,0.58,0.64	0.41,0.50,0.56,0.61
Coumarin	Toulene: diethyl ether(1:1)	10% Ethanolic KOH	Flouresent blue in uv 365nm	5	5	0.21,0.32,0.43,0.5,0.62	0.22,0.28,0.45,0.49,0.62
Flavonoids	Ethylacetate: Glaceal aceticacid: Formic acid: water(100:11:11:26)	NP\PEG	Flouresent lemon yellow in uv 365nm	1	3	0.76	0.76,0.82,0.85
Xanthones	Ethylacetate: Glaceal aceticacid: Formic acid: water (100:11:11:26)	NP\PEG	Flouresent orangish yellow in uv 365nm	1	3	0.45	0.42,0.72,0.91
Bitter principle	Ethylacetate: methanol: water (77:15:8)	Anisaldehyde-sulphuric acid reagent (AS)	Orange Brown in visible	6	9	0.12,0.26,0.42,0.54, 0.62,0.72,	0.10,0.16,0.26,0.34,0.38,0.42,0.63,0.72,0.98
Triterpenoids	Toulene: Ethylacetate (93:7)	Anisaldehydesulphuric acid reagent (AS)	Blue gray in visible	5	3	0.34,0.55,0.61,0.71,0.82	0.32,0.54,0.69
Alkaloids	Ethylacetate: methanol (90:10)	Dragendroff's reagent	Redish brown band in visible	4	4	0.18,0.32,0.51,0.82	0.16,0.34,0.52,0.64

3.1 Phenolic compounds: (fig.1 K and L)
Folin-ciocaltu is a general spray reagent used for detection and quantification of total phenolic compounds. It reacts with phenol ring to give green, blue, gray coloured compounds. In the present study microwave as well as soxhlet extract furnished similar no. (7 band) and pattern of phenolic compounds (Rf ranging from 0.25 to 0.97). Further analysis to identify specific phytochemical group by specific derivatising agent using different solvent system exhibited following results.

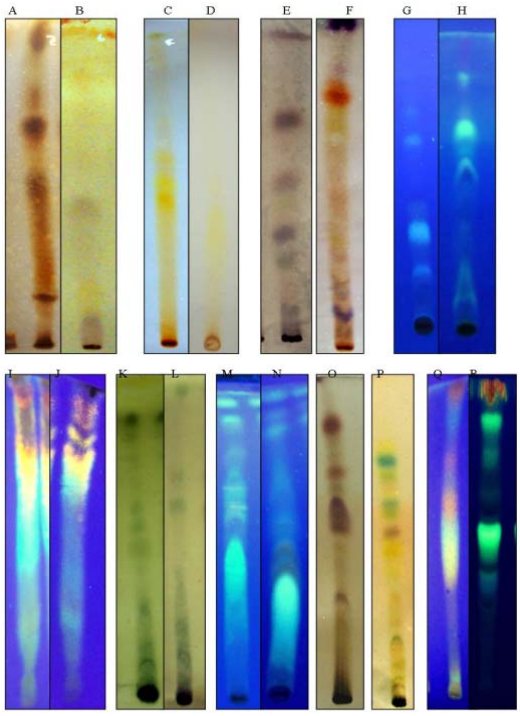

Fig 1: Comparative analysis of phytochemicals in ethanolic extract obtained by Soxhlet (soh), microwave (m). Alkaloid: A-soh, B-m, Anthracene-C-soh, D-m; Bitter principle-E-soh, F-m; Coumarin-G-soh, H-m; Flavonoid-; I-soh, J-m; Phenolics-K-soh, L-m; Phenolic acids-M-soh, N-m; Triterpenoid-O-soh, P-m; Xanthones-Q-soh, R.m.

i. Detection of Phenolic acids
Phenolic acids could be resolved satisfactory using higly polar mobile phase. Ethylacetate: methanol: water (100:13.5:10). They were detected as fluorescent bluish white colour bands after spray under in uv366 nm uv light. The no. of band differed marginally with 6 Band were from soxhlet and7 in microwave extract (fig.1 M and N). Rf ranging from 0.41to 0.91.

ii. Detection of anthracene
Anthracene derivative were detected as bright yellow coloured bands in visible light after derivatising with 10% ethanolic KOH. Both the type of extract furnished similar no. of bands (4 bands) with Rf ranging from 0.41to 0.62. (fig1. C and D).

iii. Detection of Coumarins
Coumarins are colourless compounds which can be detected in uv 365 as bright blue fluorescence. In *C. decurrens* 5 major coumarin derivative were obtained in microwave and soxhlet extract (fig.1G and H), after spraying the plate with ethanolic 10% KOH. Rf ranging from 0.21 to 0.62.

iv. Detection of flavonoids
Flavonoids are major phenolic compounds giving bright lemon yellow, green or orange coloured fluorescence. In *C. decurrens*, after NP\PEG spray, 3 bands (1 major + 2minor) in microwave and 1 major band in soxhlet extract were obtained as shown in fig4.3.I and J. Rf ranging from 0.76 to 0.85.

v. Xanthones
Xanthones are orangish yellow phenolic pigments showing similar colour reaction and Rf values to that of flavonoid. In *C. decurrens* after NP\PEG derivatization 1 band in soxhlet and 3 bands in microwave extracts were obtained (fig. Q and R).

3.2 Detection of bitter principle
Phytochemicals imparting bitterness to the plant were found to be major component of ethanolic extract in both microwave and soxhlet. However microwave could extract more no. of bitter compound (9 bands) as compared to that of soxhlet (6 bands) (fig.1E and F).The highly polar solvent system (Ethylacetate: methanol: water 77:15:8) indicate glycoside counterparts of phytoconstituent which develop brown, blue and gray colour compound after derivatising with Anisaldehyde-sulphuric acid reagent (AS).

i. Triterpenoids
In toulene: ethylacetate (93:7) mobile phase, after AS derivatization, microwave and soxhlet extract yielded distinctly different TLC profile in terms of no. and colour of bands. If violet\ blue bands are considered (typical of triterpenoid) 5 bands in soxhlet and 3 bands in microwave extract were obtained. However in Microwave, additional yellow (0.85and0.56) yellow green (0.72 and 0.63) and green bands (0.76 and 0.65) were developed. Rf value ranged from0.32 to 0.69.

3.3 Detection of alkaloids
Alkaloids were detected as reddish brown bands in visible light, after spraying the plate with dargendroff's regent. In microwave extract 1 major and 3 minor bands (fig.1 A) while in soxhlet extract 4 major bands (fig.1.B) were observed. Rf ranging from 0.16-0.82.

4. Discussion
The common problem before a phytochemist is what method and which solvent should be used for extraction of bioactive compounds? Numerous conventional (maceration, decoction, reflux, soxhlet etc.) and modern methods (ultrasound assisted and microwave assisted extraction) are available but every method (along with time and solvent of extraction) yields its own phytochemical profile.

4.1 Thin layer chromatography
The extract obtained contains vast array of bioactive secondary metabolites. To separate individual group of compounds chromatographic procedures is the most commonly used technique of the several methods (Paper chromatography, HPLC and HPTLC), thin layer chromatography (TLC) is most convenient, user friendly, flexible and faster technique which does not require high and sophisticated machinery; still the results are reliable, reproducible and this technique further can be subjected to purification of desired compounds [20, 27]. With this background, for preliminary separation and detection of phytochemical compounds in *C. decurrens* ethanolic extract was subjected to TLC technique. TLC analysis of ethanolic extract indicated that *C. decurrens* extract is very rich in different phytochemical as 4 alkaloid, 5 coumarin, 3 flavonoid, 3 xanthones, 9 bitter principle and 5 terpenoids were detected. The study revealed that mixture of high polarity solvents to be most suitable as mobile phases which included ethyl acetate, methanol and water. This finding supports the fact that 'like dissolve like' i.e to separate polar compounds, polar solvents should be used for extraction and separation [7]. In plant cells all the phytochemical groups are glycosides which are easily extracted in most polar organic solvent like ethanol. It implies that ethanol could successfully extract major polar glycosides of alkaloid, terpenoid, flavonoid, coumarin, anthracene and bitter principle. Many reports suggested an effective separation of bioactive compounds in high polarity solvents [33, 35]. Detection of major phenolic compounds namely anthracene, coumarin, flavonoids, xanthones and phenolic acids and terpenoids as well as alkaloids etc. in *C. decurrens* signify its high medicinal protential as each of these groups show a variety of pharmacological activities in human beings [28, 32].
On the basis of the fact that there are no reports on the phytochemical studies of *C. decurrens*, it was necessary to compare the phytochemical profiles of extracts obtained by different methods of extraction. For this, soxhlet extraction (conventional) and microwave assisted extraction (modern) methods were selected. In both the methods ethanol was used as common extracting solvent which is reported to be best solvent for glycosides of polar and non-polar phytoconstituents [18].

4.2 The results indicated that ethanolic extract obtained by both the methods yielded two major and one minor groups of phytoconstituents
1. **Phenolic compounds**: They were initially detected by universal phenol compounds reagent namely Folin-ciocaultu.

2. Bitter principles: The plant powder tested bitter and chemically the bitter principles were detected by AS reagent [40].

3. Alkaloids: They were detected in minor quantity with the help of a highly alkaloid specific reagent namely Dragendroff's reagent. In both the extracts cardiac glycosides, saponin and tannins were absent.
Phenolic compounds are aromatic so that they all show intense absorption in short uv range and are detected as dark absorbing bands at 254nm. Phenolic compounds were further resolved in to 5 sub-groups namely anthracene, coumarin, flavonoids, phenolic acids xanthones. Their detection was confirmed by different fluorescent colours developed before and after derivatization with specific spray reagents at longer uv range at 365nm.
It is evident that microwave method is equally efficient to soxhlet method as equal no. of bands for total phenolic compounds (7) anthracaene (4) coumarin (5) were obtained. However microwave yielded more no. of bands in case of phenolic acids (7 versus 6), flavonoids (3 versus 1) and xanthones (3 versus 1).Although no. of bands and their Rf values are significantly affected by other chromatographic condition (concentration of extract and mobile phase), method of extraction might be a contributing factor for the observed differences in phytochemical profile as other condition were kept constant.
High yield of ethanolic extract at 2 min extraction time with microwave method in *Urtica dioica* [3]. Xanthones are efficiently extracted by microwave method which show most prominent advantages over conventional heat reflux extraction in *Garcinia mangostana* [11]. They demonstrated microwave assisted method to be suitable for extraction, separation and purification of mangostins from *G. mangostana*.4 times higher efficiency of phenolic compounds than sonication extraction in 4 different spices (*Cinnamomum zeylanicum, Coriandrum sativum, Cuminum cyminum, Crocus sativus)* [12].
Application of Microwave assisted method to extract the secondary metabolite from plant material has attracted the attention of phytochemist as in Microwave assisted extraction heating of mixture (Powder +solvent) occurs in targeted and selective manner as no heat is lost. It reduces as compared to soxhlet. In case of *C. decurrens* it was only 4 minutes. Microwave assisted extraction method is most suitable for polar mixtures because the extraction is governed by ionic conduction and dipole rotation of both solid plant matrix and solvent and hence unlike soxhlet (conductive heating) microwave heat the whole sample simultaneously [41]. Microwave extraction yields are reported to be more than soxhlet because of its unique extraction principle [43]. The plants material contain traces of moisture when heated up in microwave it evaporate and generates tremendous pressure on the cell wall due to swelling of plant cell. Cell walls ultimately rupture allowing total leaching out of active constituent in the surrounding solvent [32]. The other advantages of Microwave assisted methods are
1. This methods is suitable for thermolabile constituents.
2. High sample through put as a large no. of samples can be processed in one extraction run.
3. Automation leads to accuracy, precision and reproducibility.

4. Cost saving time saving with very less solvent consumption
5. It is simple, rapid and safe.

The detailed comparative (Microwave versus soxhlet)TLC analysis in the present study led to select 3 major compounds namely xanthone (Mangiferin), flavonoid (Quercetin) and coumarin (Scopoletin) and microwave method of extraction for further HPTLC quantification.

4.3 Bitter principle

Most of the bitter principles in plant drug possess terpenoid molecule representing Secoiridoide (monoterpene, sesquiterpene, diterpene and triterpenoids). Generally mono, sesqui and diterpene are lipid soluble and occur in essential oil. The fact that *C. decurrens* is a non aromatic plant implies absence of essential oil in ethanolic extract. However glycosides of terpens eg. Secoiridoide or triterpenoid glycosides (saponin and sterol) over easily extracted in polar solvent like ethanol. In the present study 6-9 bitter glycoside derivatives were detected in both the types of extract indicting presence triterpenoid glycoside. In microwave assisted extract 3 bands. In while in soxhlet 5 grey blue band were confirmed to be triterpenoid glycosides. Interestingly the triterpenoid pattern of soxhlet extract (fig 1.O) is significantly different than that of microwave assisted (fig1.P) the addition yellow, yellow green and green bands in microwave assisted extraction extract may represent presence of Secoiridoide glycoside presence of Secoiridoide is a characteristic features of family *Gentianaceae.* A no. of Secoiridoide like amerogentin, swertiamarin, sweroside, gentiopicroside etc. have been reported in tribe *Chironae* which is closely related to tribe *Canscorinea* of family *Gentianaceae* as reported in *Swertia longifolia* [16] and in *Swertia chirayita* [24]. However presence of Secoiridoide in any of the species of *Canscora* is not known hence presence of Secoiridoide in the present study could not be confirmed.

4.4 Alkaloid

Alkaloid also contribute in bitterness of medicinal herb. An attempt to detect alkaloid in present study furnished positive result with 4 bands in both the types of extract. In literature there are conflicting reports on presence of alkaloid in *Canscora.* Certain alkaloids namely gentianine, gentiamine and gentiadine have been reported in family *Gentianaceae* eg. *Swertia* [8, 31] *C. decussata* is also reported to contain gentianine and other glycoalkaloid [1, 14]. However negative results for alkaloid. There is only one report (anonymous) of presence of alkaloid *C. diffusa* (syn *C. decurrens*) [15].

4.5 Anthracene

Anthracene (Anthranol and Anthraquinone) is a natural class of compounds which are widely applied in medicine, food and dye industry. They display antibacterial, antitrypanosomal and anti-neoplastic activity [27]. While anthraquinone compounds are best known for laxative properties [34]. In the present study 4 different anthracene derivative were detected with different Rf levels. There are no early reports on presence of anthracene, in *C. decurrens*.

4.6 Coumarin

Coumarins are considered as Phytoalexins since plants produce them as defense substance when exposed to traumatic injury like wilting or disease etc. They are recognized to posses anti-inflamatory, anti-oxidant, anti-allergic, hepatoprotective, anti-thrombic, anti-viral etc. [19]. Hence coumarin containing plants are considered as valuable therapeutic agents. In *C. decurrens,* 5 coumarin bands were detected which might contribute to its medicinal property. Although there are no reports of coumarin in *C. decurrens,* scopoletin in the other species namely *C. decussate* [36].

4.7 Flavonoids

Flavonoids are ubiquitous in plants. Most flavonoids function as antioxidant in human body [22] common examples of flavonoids are quercetin, rutin kaemeferol and myricetin [17]. The ethanolic extract furnished 3 major flavonoid bands; out of these one compounds was identified as quercetin on the basis of Rf and colour properties.

4.8 Phenolic acids

Phenolic acids are water soluble because most frequently they occur combined with sugar as glycosides. Ethanol being polar solvent could extract many phenolic acids (6-7 bands) and were detected together with flavonoids or other phenolic compounds in the present study. Some of the common phenolic acids which occur in angiosperm are hydroxyl benzoic vanillic acid, syringic acids, chlorogenic acids etc. [39]. Specific medicinal properties associated with phenolic acids are not reported in the literature but they might contribute to the common medicinal properties of phenolic compound like antimicrobial and antioxidant etc. [42].

4.9 Triterpenoids

This group includes major bioactive principles in most of the medicinal plants. Triterpenoid form basic carbon skeleton of steroid, sterol, cardiac glycoside and saponin showing masked pharmacological activities. In *C. decurrens* though terpenoidal glycosides were detected (with the help of AS, VS and LB reagents), the specific identity of saponin, sterol and cardiac glycoside was not confirmed [13]. Terpenoids namely gluanone, canscoradiane, friedelin and amyrin as well as sitosterol, campesterol and stigmasterol from *C. decussata*. Whlie in *C. perfoliata.* Azulene (Sesquiterpene) and Cedrandiol (Sesquiterpene alcohol) has and reported their anti-ulcer, anti-microbial, anti-allergic and anti-pyretic activity [38].

4.10 Xanthone

Presence of xanthone- a special type of flavonoids is a characteristic phytochemical features of Family- *Gentianaceae*. Xanthones occur in nature either in Free State or C-or o- glycosides. They may be least polar intermediate polar or strongly polar. Mangiferin is major and most polar C-glycoside xanthone reported to exhibit CNS stimulating activities [5, 36].

Mangiferin and other more than 20 xanthone derivatives are reported in *C. decussate*. In *C. decurrens* 3 major xanthones were detected in ethanolic extract [4, 35]. One of which was found to be mangiferin which positively support the earlier reports mentioned above.

5. Conclusion

Microwave extraction (4 min, 8 cycles) was more successful than soxhlet extraction method. Phytochemical analysis confirmed that the plant is very rich in terms variety of phytochemicals and number of derivatives (bands) belonging to each groups of phytochemical. Although Tribe- *Chironae*

(Family - *Gentianaceae*) exclusively contain sec -iridoide, their presence is not yet reported in genus *Canscora*. In the present investigation although their presence is indicated by large number of terpenoids, further confirmation is needed.

6. References

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