DISCUSSION
A herbal drug may be standardized on the basis of its organoleptic characters, macroscopy, microscopy, chemical constituents and/or bioactivity. In the present investigations, bioactive phytoconstituents have been made the basis of standardizing selected herbal drugs. An ideal protocol to be followed for developing procedures for standardization of herbal drugs would involve (1) use of proper analytical techniques, (2) preparation of calibration plots using reference compound (marker), (3) efficient sample preparation procedure, and (4) validation of the whole process of quantification.

### Analytical techniques

The analytical technique employed for quantification should be selective, sensitive, precise, accurate, reproducible and economical. Detection and quantification limits need not be defined where a major constituent is being determined in the test material because these limits can easily be overcome by so adjusting the concentration of sample solution as would give a response in the linear range of the calibration plot. Two analytical chromatographic techniques — HPLC and TLC, known to be far superior to non-selective analytical techniques viz. gravimetry, titrimetry and spectrophotometry, were employed for the purpose of standardization in the present investigations.

### HPLC

Although there is not much difference in the cost of reverse phase and normal phase columns, reverse phase HPLC was preferred over normal phase because of its better reproducibility and because of the fact that the solvents
used as mobile phase in the former are safer than those used in the latter. Furthermore, reverse phase columns are the most frequently used ones in HPLC. Variable volume injector permitting use of flexible injection volume and diode array detector indicating peak purity were used during trial runs for optimizing the separation of marker in the test samples. Having achieved optimization of separation, the actual analytical work was done using fixed volume injector which ensures injection of reproducible sample volumes, and UV detector which is more sensitive and gives a better linear response compared to the diode array detector.

3.1.1.2 TLC

TLC was used as an alternative to HPLC. Unlike HPLC where reverse phase system was used, normal phase was preferred in TLC as the normal phase TLC plates are cheaper than the reverse phase plates. Further, normal phase TLC offers a wider choice of mobile phase compared to reverse phase TLC. In contrast to HPLC which gave linear correlation between the concentration of analyte and the response, it was not so in case of TLC assays. The relation between concentration and response in case of TLC was linearized through optimum setting of linearizing factor during data processing. Both horizontal and vertical modes of development were used depending upon the solvent system.

The marker zones were scanned in either direction at respective $\lambda_{\text{max}}$. It is pertinent to mention here that despite $\lambda_{\text{max}}$ of lupeol/solasodine (200 nm) and madecassoside/asiaticoside (205 nm) being quite close, and the fact that absorptivity of all the four markers is low, lupeol/solasodine spots were
scanned during TLC assay without derivatization while those of madecassoside and asiaticoside had to be derivatized before scanning. This was because the concentration of madecassoside is about 16 times that of asiaticoside in *C. asiatica*, and attempts to scan spots of the two saponins in a *single run* without derivatization did not give such detector responses as could *simultaneously* be fitted in the respective calibration plots. Use of concentrated sample solution gave optimum response for asiaticoside but saturated detector response for madecassoside. On the other hand, a relatively dilute solution suited quantification of madecassoside but failed to give optimum response for asiaticoside. Since it was intended to develop an assay procedure which would quantify both asiaticoside and madecassoside in a *single run*, derivatization of the two compounds had to be resorted to in order to increase the absorptivity, thereby, increasing the sensitivity. With the increased sensitivity of the derivatized spots, it was possible to get linear detector response for both madecassoside and asiaticoside in a single run by adjusting the concentration of sample solution. Since the standardization of *C. nurvala* and *S. surattense* was based on *single* markers — lupeol and solasodine respectively, the problem of low absorptivity and, hence, low sensitivity, was overcome by adjusting sample concentrations to such levels of markers which gave optimum detector response making the assay procedure viable and reproducible.

**3.1.2 Calibration plots**

Calibration plots were prepared in order to find out the range of marker concentration which bore a linear relation with respect to the response of analytical technique. Once this range of response was established, concentrations of all test samples were so adjusted as to give a response in the established linear range.
3.1.3 Sample preparation

Sample preparation is an important step in the analysis of any test material. The sample preparation procedure is required to be simple and efficient in order to ensure maximum possible extraction of the marker and removal of interfering substances like pigments/excipients etc. Extraction procedures adopted in the present investigations were optimized systematically. Briefly, optimization procedure for sample preparation involved extraction of test material with a suitable solvent for specific periods of time, estimating the marker in the extracts thus obtained, further subjecting the test material to repeated cycles of extraction using the time period yielding optimum amount of the marker, and finally selecting the minimum number of cycles of extraction giving maximum yield of the marker.

3.1.4 Validation of assays

Any given assay procedure would remain incomplete without assessing its validity, i.e., how accurately it is able to determine the analyte in question. Expressed in different words, in order to establish the credibility of the assay procedure, we must know what proportion of the real content of analyte in the test material is being quantified by the assay. Validity of the assay was assessed by adding known amount of the marker in the test sample, subjecting the mixture to optimized sample preparation procedure, estimating the content of marker, and plotting the determined marker content against the content added. Slope of such a regression line gave an estimate of recovery of the marker, and the intercept represented the marker content in the test material. Further, it is a well known fact that if similar results are obtained by two different analytical techniques, the estimated value of the analyte is very close
to its real value in the test material. HPLC, precision and accuracy of which is well established, was applied to all the test materials. But TLC was applied to only a few representative test materials with a view to assess validity of the results.

3.2 Selection of plants and formulations

Five plants were worked up, and these were the ones with established therapeutic credibility as far as the traditional system of medicine is concerned, and on which reasonable inputs in terms of modern scientific studies have been made. *C. asiatica* is one of the most important 'Medhya Rasayana' (drugs which improve mental ability). Similarly, hepatoprotective potential of *A. paniculata* is well documented justifying its incorporation in the present investigation. Three other plants viz. *A. vasica* (bronchodilator) *S. surattense* (antiasthmatic) and *C. nurvala* (antiurolithiatic) are being investigated under the multicentric double blind clinical trial programme sponsored by ICMR with a view to validate the claims of traditional therapeutics. In addition to the aforementioned plants, the formulations which were actually used in the clinical trials or otherwise available commercially were considered worthy of incorporation in the present studies.

3.3 Selection of markers

Marker is the compound used as a reference for standardizing the test material. Ideally, the marker should be a biologically active compound, and the markers selected for standardizing the plants in the present investigation conform to this criterion. Madecassoside and asiaticoside for *C. asiatica*, andrographolide for *A. paniculata*, vasicine for *A. vasica*, lupeol for *C. nurvala* and solasodine for *S. surattense* were employed as markers.
3.4 Centella asiatica

Although qualitative and quantitative methods for analysing asiaticoside in *C. asiatica* are on records but no method of quantifying madecassoside has been reported so far. In the present studies, the plant and its formulations have been standardized on the basis of bioactive saponins madecassoside and asiaticoside, and the methods reported herein can quantify these markers in a single run.

3.4.1 Isolation and characterization of markers

*C. asiatica* whole plant was extracted with alcohol, the extract concentrated, diluted with water and partitioned successively with petroleum ether, diethyl ether, ethyl acetate and 1-butanol. Acetone soluble portion of the 1-butanol fraction gave a mixture (F1) of two saponins which when subjected to preparative HPLC gave two pure compounds, K1 and K2.

K1 was identified as madecassoside by comparing its mp, UV, IR and $^{13}$C NMR spectra with those reported in literature. Identity of K1 was further confirmed by analysis of the sugar moieties (glucose and rhamnose) in its hydrolysate. UV, IR, $^{13}$C NMR spectra and mp of K2 were observed to be comparable to those of reference asiaticoside thereby confirming the identity of K2 as asiaticoside.

3.4.2 HPLC assay

Both madecassoside and asiaticoside were efficiently resolved in a single isocratic run over a reverse phase column using acetonitrile and water (1:3) (Fig 2). Methanol-water (1:3) and acetonitrile-water (1:3) were used
respectively for preparative and analytical work. Although both the solvent systems were equally efficient, acetonitrile (UV cut-off λ190 nm) was preferred over methanol (UV cut-off λ210 nm) because of better UV transparency. This higher transmitting property of acetonitrile helped increase sensitivity of the assay procedure. Selectivity (α) and resolution (R) factors for madecassoside (Rt = 11.7 min; k' = 4.8) and asiaticoside (Rt = 21 min, k' = 9.5) were observed to be 1.98 and 4.8 respectively.

Calibration plot of madecassoside was observed to be linear over the range 2-40 µg while that of asiaticoside correlated linearly over the range 0.2-4.0 µg (Table 5). The regression equation for madecassoside and asiaticoside were 
\[ y = 110.38x + 30.97 \text{ and } y = 946.23x + 25.63 \] respectively, and the goodness of regression fit was highly satisfactory for both the plots as suggested by high value (0.999) of coefficients of determination (Fig 1).

The optimum procedure for preparing samples of test material for quantification of madecassoside and asiaticoside was observed to be three extraction cycles each of 30 min (Table 6). Percentage recovery of madecassoside and asiaticoside was found to be 94 and 92 respectively (Fig 3).

Madecassoside was found to be the major saponin of *C. asiatica* with concentration being about 13 times than that of asiaticoside (Table 8). Though there was no statistically significant difference in the asiaticoside content of the whole plant procured from different sources, madecassoside content in the Dabur sample was significantly lower than the samples of plant procured from Herba Indica and those collected from wild source (Table 8). *C. asiatica*
leaves were found to contain the highest amount of both the glycosides and the roots contained the minimum. Madecassoside and asiaticoside contents in Mentat tablets were found to be 1.62% and 0.225% respectively (Table 8). Thus, each Mentat tablet (mean weight 714 mg) contains 11.6 mg of madecassoside and 1.6 mg of asiaticoside respectively.

Since both Shankhpushpi and Brahmi Ghrit contain various plant extracts (Table 2) in soluble form, these were not subjected to the usual optimized extraction procedure for sample preparation (section 2.2.2.2) but were processed directly to get rid of the excipients. The sugar in Shankhpushpi was precipitated out by adding excess of methanol while clarified butter (Ghee) from Brahmi Ghrit was removed by dissolving the formulation in hexane and partitioning out the saponins with methanol.

3.4.3 TLC assay

Efficient baseline separation of madecassoside (hRf 37) and asiaticoside (hRf 47) was achieved on HPTLC plates by horizontal development in 'sandwich' configuration using solvent system S1 (Fig 5). This configuration allowed simultaneous use of either end of the chromatoplate, and cut down usual development distance of 70-80 mm to 40 mm thereby economizing on both the time and material. Marker zones were scanned, after derivatization, at 605 nm. Calibration plots for madecassoside and asiaticoside were linear over the range 0.3-4.80 µg and 0.03-0.48 µg respectively (Table 9; Fig 4). The regression equation for madecassoside was: \( y = 5533.97x + 61.17, r^2 = 0.998 \) and for asiaticoside was: \( y = 28630x - 61.5, r^2 = 0.998 \). Validity of the assay is suggested by high recovery percentages — 94 (madecassoside) and 90
(asiaticoside) (Fig 6). Madecassoside and asiaticoside contents in *C. asiatica* whole plant and Mentat tablets were found to be 3.65% & 0.22% and 1.54% & 0.21% respectively (Table 11), and there was no statistical difference between these values and those obtained by HPLC assay (Table 8).

TLC assay was found to be about 6 times more sensitive than the HPLC assay (Fig 1 and 4) because of derivatization of the saponin spots in case of the former which led to increased absorptivity. On the other hand, higher $r^2$ value in case of HPLC assay (0.999) as compared to TLC assay (0.998) indicated better precision of the HPLC assay (Fig 1 and 4). The fact that the two techniques gave similar results confirms the accuracy and validity of the assay procedure developed for determination of the bioactive saponins in *C. asiatica*. Furthermore, either technique can be applied for standardizing polyherbal formulations containing *C. asiatica*.

### 3.5 *Andrographis paniculata*

#### 3.5.1 HPLC assay

The hepatoprotective activity of *A. paniculata* has been attributed to its major diterpenoid lactone andrographolide which was employed as marker for standardizing the plant and its formulations using reverse phase HPLC system. The marker resolved at Rt 11.8 min using methanol-water (47:53) as mobile phase (Fig 8). The calibration plot showed a linear relation of andrographolide amount in the range of 0.1-5.0 μg (Table 12; Fig 7). Regression equation for the plot was calculated to be $y = 1119.52x -17.47$ ($r^2 = 0.999$). Maximal amounts of andrographolide were extracted from test materials using three cycles each of 60 min reflux (Table 13), and the assay showed an overall recovery of the marker to be 91% (Fig 9).
Samples of *A. paniculata* whole plant/leaves and Livotone capsules were prepared following the optimized procedure (section 2.3.2.2) while Kalmegh compound (liquid formulation), diluted with methanol, was directly subjected to HPLC. Adrographolide content in the leaves (2.31%) was higher than in the whole plant (0.75%) (Table 15). Each Livotone capsule quantified for 3.2 mg (0.43% w/w) andrographolide, and the content in Kalmegh compound was observed to be 1 mg/ml (0.10% w/v).

Earlier, a normal phase HPLC system for standardizing *A. paniculata* on the basis of andrographolide content has been reported from our laboratory.\(^{147}\) Though this method is as sensitive, precise and reproducible as the reverse phase HPLC assay reported herein but the latter has a distinct advantage for determining andrographolide in aqueous-base formulations. Such formulations can be directly analysed on a reverse phase system bypassing the usual time consuming and tedious process of extraction. Furthermore, cost factor and disposal problem of chloroform (one of the components of mobile phase in normal phase HPLC) is also done away with while using reverse phase HPLC which uses methanol-water as the mobile phase.

### 3.5.2 TLC assay

Andrographolide spots appeared at mean hRF 59 on normal phase TLC plates developed in solvent system S4 (Fig 11). In contrast to 50 folds linearity range of andrographolide in HPLC assay, its linearity range was only 18 folds (Table 16; Fig 10) in the TLC assay. The slope and intercept of the regression line were 20101.62 and 1194.77 respectively with \( r^2 \) equal to 0.998. Recovery (89%) of the marker in TLC assay (Fig 12) was almost similar to that in HPLC.
assay. The results of andrographolide estimation by TLC (Table 18) and HPLC (Table 15) assay did not vary statistically confirming validity of both the assays.

It would be pertinent to mention here that though the Indian Pharmacopoeia\textsuperscript{267} specifies a minimum of 1\% w/w of andrographolide in Kalmegh (\textit{A. paniculata}), its actual content is less (0.75\%). The pharmacopoeial method for determining andrographolide is gravimetric which does not involve purification of andrographolide and, therefore, records a higher content because of associated impurities.

3.6 \textit{Adhatoda vasica}

\textit{A. vasica} and its formulations have been standardized on the basis of the major bronchodilatory and expectorant pyrroloquinazoline alkaloid vasicine which was isolated from the leaves of \textit{A. vasica} following the usual alkaloid extraction procedure, and characterized by mmp, co-TLC and comparing its UV and IR spectra with those of the authentic reference sample of vasicine. Though gravimetric,\textsuperscript{172} titrimetric\textsuperscript{188} and spectrophotometric methods\textsuperscript{189} have been reported for the analysis of vasicine but these being devoid of selectivity can not be applied to polyherbal formulations.

3.6.1 \textbf{HPLC assay}

Vasicine was resolved over a reverse phase HPLC system using isocratic mobile phase, and detected at $\lambda_{298}$ nm (Fig 14). The detector response observed a linear relation to the vasicine content in the range 0.05-0.80 $\mu$g (Table 19; Fig 13). Regression equation for the calibration plot was:
y = 10666.97x - 32.2 (r^2 = 0.999). Three cycles of refluxing (each of 1 h) the test materials with methanol were observed to afford optimum extraction of the marker (Table 20). Recovery of marker calculated after spiking the test material with known amount of the marker was found as high as 94% (Fig 15). While all the solid test materials viz. *A. vasica* leaves/stem, Shereeshadi Kashaya and Yastyadivati were extracted with methanol and their methanol extracts processed as per the optimized procedure for sample preparation (section 2.4.2.2), the liquid formulations Glycodin, Zefs and Vasaka syrup IP were directly subjected to the optimized sample preparation procedure skipping the first step of extracting with methanol as these contain various ingredients in solution form. *A. vasica* leaves were found to be rich in vasicine content (1.37%) (Table 22). In contrast, *A. vasica* stem contains only 0.0134% vasicine. Shereeshadi Kashaya which is prepared from the extracts of four plants, and Yastyadivati which represents a mixture of nine plants (Table 2) were found to contain respectively 18.1 mg and 0.71 mg of vasicine per 100 g. Though Vasaka syrup is a pharmacopeial preparation, the pharmacopeia mentions only its method of preparation and is silent about its assay. The reverse phase HPLC assay reported herein was applied to Vasaka syrup IP, and its mean vasicine content was determined as 20.36 mg/100 ml. Vasicine content of commercially available expectorant, Glycodin and Zefs (polyherbal) was found to be 2.19 mg% and 2.42 mg% w/v respectively.

### 3.6.2 TLC assay

Well resolved peaks of vasicine appeared at hRf 51 on normal phase HPTLC plates developed in solvent system S5 with detection being done at λ298 nm without derivatizing vasicine zones (Fig 17). Linear relation between
the detector response and vasicine content was observed in the range of 0.25-4.00 μg (Table 23; Fig 16). Regression equation for the plot was calculated to be \( y = 11359.45x + 1142.37 \) \( (r^2 = 0.998) \). A simplified sample preparation procedure was adopted for TLC assay. The solid test materials, refluxed with alkaline methanol using three cycles each of one hour (Table 24), were subjected directly to TLC analysis bypassing the alkaloid purification steps employed in HPLC assay. However, because of low concentration of vasicine in Glycodin (liquid formulation), its sample for analysis had to be enriched by adopting alkaloid extraction procedure. The assay showed a recovery of 92% of the marker (Fig 18) which was almost akin to that observed for HPLC assay (94%) (Fig 15). Vasicine content determined by TLC (Table 26) and HPLC (Table 22) did not show statistically significant variations suggesting that the two assays are valid.

3.7 *Crataeva nurvala*

*C. nurvala* stem bark and dried aqueous decoction (Varun formulation) prepared from the bark have been standardized on the basis of major antiurolithiatic principle lupeol. Lupeol was isolated from the petroleum ether extract of the stem bark. Concentration of the extract yielded lupeol in crude form which was purified by repeated crystallization. Identity of the isolated lupeol was confirmed by mmp, co-TLC and comparing its UV and IR spectra with those of the authentic reference sample of lupeol. Although a lot of work has been done on the phytochemical and bioactivity aspects of *C. nurvala* but no analytical work has been reported so far on the plant.
3.7.1 **HPLC assay**

Well defined peaks of lupeol (Rt = 6.6 min) could be resolved by isocratic mixture of 2-propanol and water over a μBondapak C₁₈ column (Fig 20). A six point calibration plot of lupeol was found to be linear over the range 0.312-10.00 μg (Table 27; Fig 19). Linear least squares equation for the plot was calculated to be \[ y = 9332.47x - 598.44 \] \((r²= 0.999)\). While standardizing the extraction procedure for sample preparation it was observed that optimum extraction of the marker from test materials required three cycles (each of 60 min) of reflux with hexane (Table 28). Recovery level of the marker was equal to 90% (Fig 21). Mean lupeol content in the stem bark of *C. nurvala* and Varun formulation was found to be 0.60% and 0.02 % w/w respectively (Table 30).

3.7.2 **TLC assay**

Well defined spots of lupeol appeared at a mean hRf 39 over silica gel plates developed with *n*-haxane-ethyl acetate (9:1) (Fig 23). Unlike the calibration plot for HPLC assay which was linear over the range 0.312-10.00 μg, linearity range of the plot in TLC assay was 0.312-5.00 μg (Table 31), i.e., two fold less than that for the HPLC assay. Regression equation for the plot was found to be \[ y = 5576.95x + 568.7 \] with \(r² = 0.999\) (Fig 22). Recovery (90%) of the marker by TLC assay (Fig 24) was same as that by the HPLC assay. Statistically insignificant difference in results obtained by TLC (Table 33) and HPLC (Table 30) confirmed validity of the two assay procedures.
3.8 *Solanum surattense*

Though a number of reports 241-251 have appeared on qualitative and quantitative analyses of solasodine in various *Solanum* species but none deals with *S. surattense* or its herbal formulations. The plant and its formulation were standardized on the basis of bioactive compound solasodine using HPLC and TLC.

3.8.1 HPLC assay

The marker solasodine was resolved isocratically using tris buffer and acetonitrile over a reverse phase column (Fig 26). The calibration plot observed a linear relation of solasodine amount in the range 2-16 µg (Table 34). Regression equation for the plot was calculated to be: $y = 764.81x + 9.74$ ($r^2 = 0.999$) (Fig 25). Optimum extraction of the marker from test material was achieved by subjecting it to two cycles of refluxing, each of 60 min, with acidified methanol followed by acid hydrolysis and usual alkaloid extraction procedure (Table 35). The assay showed an overall recovery of 90% (Fig 27). Maximum amount of solasodine was present in the fruits (2.80%) and minimum in the roots (0.07%) of *S. surattense* (Table 37). Dried aqueous decoction of the whole plant (Kantakari formulation) quantified for 0.39% solasodine.

3.8.2 TLC assay

Normal phase silica gel TLC plates were used for resolving solasodine (hRf 52) using chloroform-methanol (Fig 29). The chromatoplates were scanned without derivatization at $\lambda 200$ nm. Calibration plot of solasodine was linear over the range 5-40 µg (Table 38). Regression equation for the plot was $y = 374.62x + 178.63$ ($r^2 = 0.999$) (Fig 28). Recovery of the marker by TLC assay was 90% (Fig 30), and this matched with that by HPLC assay.
Solasodine content in *S. surattense* whole plant and Kantakari formulation was found to be 0.19% and 0.36% respectively (Table 40), and there was no statistical difference between these results and those obtained by HPLC assay (Table 37) confirming validity of the two assays.