CHAPTER 5

DISCUSSION
5.1 Morphological study

Aroids are the most promising starch source in the tropical regions of the world. The energy entrapment done by the aroid plants was higher than that of rice and other starchy plants. The morphology of the aroid is always confusing and most of the time the local name is taken into account to designate the cultivated varieties. This is also important for researchers dealing with starch of aroids and other root crops, as because the physico-chemical properties of starch get affected by the environmental factors like temperature, humidity, rain fall, soil type etc. On the basis of morphological characters (Table 4.1) and coloured photograph of the aroids (Figure 4.1 and 4.2), the stem of the species *C. esculenta* was found to be swollen. The outline of the leaf is broadly ovate, peltate, cordate, head pendate from the erect petiole, apex obtuse, basal lobe round; lateral veins prominent on the lower surface, distal side veinlet of primary lateral veins arcing towards margin and forming parallel regular sieves. Petiole junction of both aroids was found to be not at the blade border. The *X. caramu* leaf showed a narrow ‘V’ shape depression as compared to *C. esculenta* along with a less triangular tendency. The height of *X. caracu* was also found to be lesser to *C. esculenta*. The corm of *C. esculenta* was unique in having multiple petiole origins or fusiform corms as described by Lakhanpaul et al. Corm morphology of *X. caracu* was similar to *X. sagittifolium* but the lower part was tapering. *X. sagittifolium* and *X. caracu* possessed short and stout aerial stem that exude milky sap on being cut or injured. Leaf is rosulate, blade sagittate, deep green above, horizontal to slightly pendent (not vertically pendent). The petiole is connected to the leaf at the border i.e. at the bottom of the ‘V’ shaped depression. Two secondary veins are more prominent as compared to *Colocasia* species. The morphology of *A. paeonifolius* was quite different from the other three species; its single petiole rose up from the upper central depression of the corm. The petiole continued up to the leaf apex like other aroids but before that it trifurcated. Each trifurcation again bifurcated two-three times followed by the lamellae of leaf to give a false individual leaf appearance. Corms of all four species are the modified stem that stores starch.
They also act as organs of regeneration to tide over the unfavorable conditions. The morphology of *Xanthosoma* species was described by Castro *et al.*\(^{221}\). They described the growth conditions of *X. violaceum*, height at maturity being 62.9-111.4 cm was found to be shorter than *X. caracu* (80.25 - 135.60 cm) and *X. sagittifolium* (84.43 - 116.60 cm). In the present study, plants of *Xanthosoma* species were found to be bigger. This could be due to the growing conditions as mentioned by soil type, availability of sunlight etc. The number of petiol or leaves in *X. violaceum* was reported to be 3.6-5.1 which was found to be 3.4-4.1 in the present *Xanthosoma* species. The leaf surface area of *X. violaceum* was found to be higher (674-1479 cm\(^2\)) as compared to the observed value of 1213.1 - 1533.63 cm\(^2\) in *X. caracu* and 956.23- 1936.75 cm\(^2\) in *X. sagittifolium* (Table 4.1). The variation in leaf area of *X. violaceum* could be ascribed to the different strains as studied by Castro *et al.*\(^{221}\). Boyce *et al.*\(^{222}\) described the morphological features of *Araceae* with generic keys. Their description for the genus *Amorphophallus* matched with the observed results for the present study with special reference to conspicuous and snake-skin like markings of petiole and leaf lamina.

### 5.2 Biochemical characterization

The moisture content of the aroid corms were found to be in the order of *A. paeonifolius*\(>\) *X. caracu*\(>\) *X. sagittifolium*\(>\) *C. esculenta* (Table 4.2). In general, the moisture content was found to be higher as compared to reports by earlier authors. This could be attributable to the geographical location. High moisture content is a problem in post harvest storage of corms. The ash content was in the order of *X. sagittifolium* \(>\) *C. esculenta* \(>\) *A. paeonifolius* \(>\) *X. caracu*. The crude protein content was in the order of *C. esculenta* \(>\) *A. paeonifolius* \(>\) *X. sagittifolium* \(>\) *X. caracu* where as the crude lipid content *C. esculenta* \(>\) *A. paeonifolius* \(>\) *X. sagittifolium* \(>\) *X. caracu*; starch content *C. esculenta* \(>\) *X. caracu* \(>\) *X. sagittifolium* \(>\) *A. paeonifolius*; crude fiber content *X. caracu* \(>\) *A. paeonifolius* \(>\) *X. sagittifolium* \(>\) *C. esculenta*; and soluble sugar content *C. esculenta* \(>\) *X. sagittifolium* \(>\) *X. caracu* \(>\) *A. paeonifolius*. The species *C. esculenta*. 


*esculenta* was found to be promising. The starch and protein content of the aroid corms ranged from 11-20% and 1-3%, respectively. In the case of corms assayed, *Amorphophallus* species was found to contain lesser than the average starch and protein. The granule size of *C. esculenta* starch was observed to be smaller as compared to earlier reports indicating more suitability of its starch for the various nutritional applications\(^223\). Maximum variation in the starch granule size was observed in the case of *Xanthosoma* species as reported by several workers. As compared to other species, the starch was spherical in the case of *C. esculenta*.

In *A. paeoniifolius* moisture content was 81.64 ± 3.49%, Na 33.91 ± 0.18 mg/100 gm (dry wt), K 40.91 ± 1.69 mg/100 gm (dry wt), Ca 3.76 ± 0.17 mg/100 gm and the largest starch granule size of 4.86 - 7.20 \(\mu\)m. The highest content of ash was estimated in *X. sagittifolium* (3.91 ± 0.16%) and crude fiber (1.98 ± 0.05%) in *X. caracu*. In the case of *C. esculenta* contents of crude protein 2.89 ± 0.04%, crude lipid 1.24 ± 0.03%, starch content 14.81 ± 0.60%, soluble sugar 1.80 ± 0.06%, net energy 103.25 cal/100 gm (fresh weight) and Mg 1.44 ± 0.07 mg/100 gm (dry weight) were the highest. The moisture content of *X. sagittifolium* corm was found to be 80.36±3.26% against the earlier report of 69.81±0.11%\(^224\). There was variation in the other biochemical compositions of *X. sagittifolium*, the crude protein content also varied to 1.261±0.10% against the reported value of 2.83±0.30%. The moisture content of the apical part of *X. sagittifolium* was reported to be 77.4% which was also found to be lower as compared to the moisture content estimated in the present study\(^225\). The high moisture content of the corm might be due to the environmental effects like growth condition, soil type, rainfall etc. The protein, lipid, ash and starch content of *X. sagittifolium* corms were also found to be lower as compared to the earlier reports\(^8\). The moisture content of *C. esculenta* was also found to be lower; where as the average crude lipid content of the species was higher 1.24±0.03%. The ash content of *C. esculenta* (2.86 ± 0.08%) was comparable to the reported value of 1.88%\(^225\). The mineral content of *C. esculenta* and *X. sagittifolium* including K was found to be low, where as Na comparable. Huang
et al.\textsuperscript{226} reported moisture content of \textit{C. esculenta} to be 63.6-72.4\% as compared to the present value of 77.25 \( \pm \) 3.42\%. The starch content was found to be 14.81 \( \pm \) 0.60\% against the reported value of 21.1-26.1\%.

5.3 Karyotyping
The active cell division of \textit{C. esculenta} was half an hour earlier to the other aroids. The maximum number of metaphase cells was observed for all four aroids during their maximum active cell division period (Figure 4.3). The number of chromosomes observed in the aroid species was 2\textit{n}=28 in \textit{A. paeoniiifolius} and \textit{C. esculenta} whereas in \textit{X. caracu} and \textit{X. sagittifolium} 2\textit{n}=26. Parvin \textit{et al.}\textsuperscript{227} described karyotype of seven varieties of \textit{Colocasia esculenta} from Bangladesh. The somatic chromosome complement as arranged in the present study showed the basic number to be \( x=7 \), which was also supported by Parvin \textit{et al.}\textsuperscript{227}. It suggested that \textit{C. esculenta} is a tetraploid (2\textit{n}=28). In \textit{A. paeoniiifolius} the arrangement suggests that the species is diploid (2\textit{n}=28) with basic chromosome number \( x=14 \) and is also supported by the earlier report of Chauhan and Brandham\textsuperscript{114}. The basic chromosome number of \textit{X. caracu} and \textit{X. sagittifolium} was found to be \( x=13 \) and the same (\( x=n=13 \), 2\textit{n}=26) was also reported by Marchant\textsuperscript{124}.

5.4 Molecular biological study
Gel electrophoresis data of genomic DNA (Table 4.4 and Figure 4.5) of all four aroid species established the yield of quality DNA\textsuperscript{206}. The yield of DNA of all four aroids was in the order of \textit{X. sagittifolium} > \textit{A. paeoniiifolius} > \textit{C. esculenta} > \textit{X. caracu}. The species \textit{X. sagittifolium} showed the highest yield of quality DNA.

The genome size of the aroids was in the order of \textit{C. esculenta} > \textit{X. caracu} > \textit{X. sagittifolium} > \textit{A. paeoniiifolius} (Table 4.5). The genome size of the species \textit{C. esculenta} was found to be 14.1 pg and it would be between 2C-3C value as given by Bennett and Leitch\textsuperscript{228} for \textit{C. antiquorum}. They also reported 1C value of \textit{X. sagittifolium} to be 8.8 pg, which is 0.76 pg higher than that of
the present investigation (Table 4.5). Genome size of *A. paeoniifolius* (5.04 pg) recorded in the present investigation was higher as compared to the earlier report of 4.2 pg\(^{228}\). There are very few reports on the genome sizes of *Xanthosoma* species.

The primer OPW-15 possessed sequence complementation *C. esculenta* (Figure 4.7) genome, but absence of complementation of the primers OPW-15 and OPW-16 sequences with that of *A. paeoniifolius*. Amplification of genomic DNA using six primers yielded 25 reproducible RAPD loci for an average of 4.16 bands per primer; 21 (84\%) of them were polymorphic and 4 (16\%) were monomorphic. Among *Xanthosoma* species only four bands (16\%) were polymorphic with the rest of polymorphism existing between *Colocasia* and *Amorphophallus* species (Figure 4.7, Table 4.6).

The primers were selected on the basis of their polymorphism in Indian *Colocasia* cultivars\(^{112}\). The results showed that both *Xanthosoma* species are closely related to each other (66\%) as compared to their similarity to others (Table 4.7). *Amorphophallus* species was found to be 50\% similar to both *X. caracu* and *C. esculenta* but dissimilar to *X. sagittifolium*. Hence, it could be concluded that *X. caracu* and *C. esculenta* species are equally distant from *A. paeoniifolius* species. *C. esculenta* used in the study might be the same morphotype M-5 having ID no. TC5 used for RAPD analysis by Lakhanpaul *et al.*\(^{112}\). The relation between the species was properly understood from the dendrogram prepared by feeding the data generated from the RAPD (Figure 4.8). In the dendrogram *X. caracu* and *X. sagittifolium* were found to be closer (100\%) as compared to completely separate species of *C. esculenta*. *A. paeoniifolius* related to two *Xanthosoma* species by 64\%.

In the study, an effort was made to estimate the genetic similarity and dissimilarity among the edible aroid species at the molecular level. The primers (OPW-04, OPW-05, OPW-08, OPW-10, OPW-15 and OPW-16) in the investigation were extensively used to characterize *C. esculenta*. The primers used provided reliable polymorphism in *A. paeonifolius*. Primers selected for the study showed 88.9-100.0\% polymorphism in the case of Indian *C. esculenta*.
morphotypes, which was also reported by Lakhanpaul et al.\textsuperscript{112}. The genome diversity analyses of aroids with a limited number of genotypes were not enough to prepare a dendrogram. A number of morphotypes with more primers would be needed for the same.

The genome size of the edible species was determined using a comparatively easy and established method than prevalent expensive methods like fuelgen densiometry and flow cytometry. At present, flow cytometry is becoming a popular method in the determination of genome size. The method needs the establishment of the sophisticated costly equipment, flow cytometer. The cost is indeed prohibitive for most of the organizations and the handling of the equipment needs the expert manpower. On the other hand, discrepancies are observed in flow cytometry data of a single sample analysed in different laboratories. Hence, an effort was made by Konwar \textit{et al.}\textsuperscript{131} to develop a novel but simple and less expensive method for the determination of genome size of plants without compromising the quality. With the collection of suitable tender leaves from the raised corms of the selected edible aroids, quality genomic DNA was isolated\textsuperscript{131}. The protocol could yield quality DNA as evident from the UV-Vis spectrophotometric absorbance ratio at 260:280 nm being 1.735-1.916 (Table 4.7). Two critical points were taken into account in this method; firstly, the method for the isolation of genomic DNA was such that it could isolate almost all the DNA from the nuclei, and secondly the accurate determination of the intercellular space in the aroid species. The developing countries on one hand have vast resources of plant biodiversity while on the other hand face the problem of acquisition and maintenance of Flowcytometers\textsuperscript{229}. Hence, the present method could be of great help for the researchers of developing countries having limited access to the facilities like Flowcytometer.

5.5 Compound purification

5.5.1 Total polyphenol content

The total polyphenol content (TPC) extracted from the aroid corms was estimated and data obtained are presented in Figure 4.9. The solvents were
100% and 80% MeOH and water. The TPC of aroids were in the order of *A. paeonifolius* (0.303±0.02 mg/100 g) > *C. esculenta* (0.21±0.03 mg/100 g) > *X. caracu* (0.16±0.06 mg/100 g dry corm) > *X. sagittifolium* (0.11±0.02 mg/100 g) for gallic acid equivalent (in 80% MeOH extract); *A. paeonifolius* (0.203±0.04 mg BHT and 1.373±0.12 mg tannic acid/100 gm dry corm) > *X. sagittifolium* (0.14±0.08 mg and 0.59±0.05 mg) > *C. esculenta* (0.14±0.02 mg and 0.51±0.08 mg) > *X. caracu* (0.10±0.04 mg and 0.16±0.06 mg) for BHT and tannic acid equivalent (in 80% MeOH extract); *A. paeonifolius* (1.896±0.10 mg/100 gm dry corm) > *X. caracu* (0.86±0.05 mg/100 gm) > *X. sagittifolium* (0.82±0.04 mg/100 gm) > *C. esculenta* (0.70±0.07 mg/100 gm) for quercetine dihydrate equivalent (in 80% methanol extract). In the absolute methanol extract, gallic acid and BHT possessed the same trend like quercetin equivalent in 80% methanol extract; tannic acid and quercetin dihydrate equivalent in the absolute methanol extract were not detected. In the water extract, the total polyphenol equivalent was in the order of *A. paeonifolius* > *X. caracu* > *X. sagittifolium* > *C. esculenta* for all four standards.

### 5.5.2 Total flavonoid estimation

In view of the significant polyphenol content in water and 80% methanol extracts (Figure 4.10), the absolute methanol extract was not considered for the total flavonoid estimation. The flavonoid content (quercetin equivalent) followed the order *A. paeonifolius* (62.26±3.51 mg/100gm dry corm) > *X. caracu* (9.65±1.64 mg/100gm) > *X. sagittifolium* (5.89±0.86 mg/100gm) > *C. esculenta* (not detected) in 80% methanol extract and *A. paeonifolius* (178.71±12.22 mg/100 gm) > *C. esculenta* (142.39±11.94 mg/100 gm) > *X. caracu* (93.54±7.85 mg/100 gm) > *X. sagittifolium* (86.02±5.55 mg/100 gm) in the water extract. The flavonoid content in the water extract was significantly higher as compared to 80% methanol. Both solvent extracts were used for the blood clotting experiment.
5.5.3 The antioxidant capacity

The corm extracts were used for assaying antioxidants. The order of the antioxidant capacity was *X. caracu* > *A. paeonifolius* > *X. sagittifolium* > *C. esculenta* in the case of 80% methanol extract and *A. paeonifolius* > *X. caracu* > *C. esculenta* > *X. sagittifolium* in the case of water extract. The water extract of *C. esculenta* and *X. sagittifolium* were similar in their antioxidant activity (Figure 4.12).

5.5.4 Blood clotting

With the exception of *C. esculenta* in 80% methanol, other corm extracts were found to enhance the process of blood clotting, whereas, water extract of all aroid corms were found to be blood clotting inhibitor (Figure 4.13).

The total polyphenolic content (0.44 ± 0.09 mg gallic acid equivalent/100 gm dry weight) and flavonoid content (178.71 ± 12.22 mg quercetin equivalent/100 gm dry weight) of water extract of *A. paeonifolius* were recorded to be the highest. Blood coagulation enhancing property was the highest in *X. sagittifolium* 80% methanolic extract (18.1 sec.). The DPPH scavenging property was the highest in *X. caracu* (19.00 ± 1.54%). Non-detection of flavonoid content in 80% methanol extract of *C. esculenta* could be attributed to the high response of quercetine dihydrate to the estimation method and the occurrence below the detection level of flavonoid in the aroid.

Flavonoids are the molecules which are not only known for their antioxidant activity, but also provide health benefits against cancer and heart diseases. Blood clotting was tested to investigate the scientific ground for the traditional practice of applying aroid sap in wounds as done by the people of the North Eastern India. The positive results with 80% methanol extracts of *Amorphophallus* and *Xanthosoma* species opened the prospect for further study of activity in *vivo*. On the other hand, the tribal people use water for grinding corms to prepare the extract for applying to wounds. This suggests that there is a need to study their preparation process carefully as well as in *vivo* study for evaluating the exact wound healing property.
The polyphenol content in water extract followed the pattern of the antioxidant activity as described above (Figure 4.9 and 4.12). The water extract of four edible corms though showed the presence of maximum polyphenols and flavonoids, but possessed poor activity, except for *A. paeonifolius*. In the case of *X. caracu*, 80% methanol extract showed 19.00±1.54% scavenging of DPPH-stable free radical which was maximum among the corms and types of extracts with 1,479.48 mg in every 100 gm dry corm powder, as calculated from the standard curve of antioxidant activity of quercetin dihydrate. The observed antioxidant activity of the extracts might be due to the neutralization of free radical character of DPPH.

5.5.5 Antimicrobial activity

5.5.5.1 Antibacterial activity

All extracts were used for the determination of the antibacterial activity against *E. coli* and *S. aureus*. In the case of extracts loaded with 80% methanolic extract showed small zones for both bacteria. The other two types of extracts i.e. water and methanol only extracts did not show any activity against these two tested bacteria (Figure 4.14).

5.5.5.2 Antifungal activity

Eighty percent methanolic extract and water extracts were used to test antifungal activity against *F. oxysporium* and *C. albicans*. The *X. sagittifolium* 80% methanol, water only extract showed activity against both the tested fungi. The water only extract of *C. esculenta* was found to be positive against both the tested fungi. *A. paeonifolius* water only extract was found to be positive against both the tested fungi. All other extracts were found to be negative against the tested fungi (Figure 4.15).

5.5.6 Thin layer chromatography (TLC)

On the basis of high polyphenolic content of 80% methanol extract in water and its positive results in antibacterial and antifungal study, it was
concluded that the polyphenolic compounds might be responsible for the antimicrobial activity. Taking this into account the polyphenolic compounds were accessed in 80% methanolic extract; and as such the other two extracts were discarded. TLC of 80% methanol-water extract was done to separate the phenols (Figure 4.16).

5.5.7 Column chromatography

Column chromatography was done using the same solvent as used for developing TLC with a hope of partial purification of the crude extract. The fractions were profiled with respect to their polyphenol content. The highest polyphenol containing fractions were collected for further purification using HPLC (Figure 4.17). For *A. paeoniifolius*, fractions 9 (AC-I) and 31 (AC-II); *X. caracu*, fractions 10 (BC-I) and 31 (BC-II); *C. esculenta* fractions 15 (CC-I) and 29 (CC-II); and for *X. sagittifolium* fraction 19 (XC-I) were taken for further purification as they were found to contain the highest polyphenolic content.

5.5.8 High Performance Liquid Chromatography (HPLC)

The peak generated from HPLC purification was presented in Table 4.8. The HPLC profile of the AC-I (Figure 4.18) fraction showed 2 prominent peaks which were collected by repetitive injection and evaporated to dryness. The collected peaks were labelled as AC-I (1) and AC-I (2). From AC-II fraction also two prominent peaks were collected and labeled as AC-II (1) and AC-II (2). Similarly, from *X. caracu* the collected fractions were labelled as BC-I (1), BC-I (2) and BC-I (3); BC-II (1) and BC-II (2). *C. esculenta* fractions collected from HPLC were labeled as CC-I (1), CC-I (2), CC-I (3) and CC-I (4); CC-II (1), CC-II (2), CC-II (3), CC-II (4) and CC-II (5). The single peak collected from the single fraction of *X. sagittifolium* was labeled as XC-I (1).

Some of the above mentioned HPLC fractions were not collected as the amounts were not sufficient to trace, as such was not possible to proceed for further analysis. These fractions were AC-I (2), AC-II (2) and CC-II (5). The
other fractions were subjected to antimicrobial study, FT-IR and NMR (H\(^1\) and C\(^{13}\)).

5.5.9 Fourier transform Infrared spectroscopy (FTIR)

The results obtained from FT-IR peak analysis were presented in Table 4.9. All fractionated compounds contained at least a benzene ring which was confirmed by the FTIR spectral pattern (Figure 4.20 and 4.21). The broad peaks left to 3,000 cm\(^{-1}\) might be attributed to the stretching vibration of the bonds between an aromatic ring and H atom. The peaks ranged from 1,400-1,500 cm\(^{-1}\) and 1,585-1,600 cm\(^{-1}\) might be ascribed to the C=C stretching of an aromatic ring. The peaks between 660-1,000 cm\(^{-1}\) might be due to the loop bending of R=C-H of an aromatic ring.

The peaks between 3,231-3,657 cm\(^{-1}\) of AC-I (1) and XC-I (1) might be due to the O-H bond stretching of –COOH group associated with the aromatic ring. The peaks might get broadened due to superimposition with the C-H stretching peaks of aromatic ring. The strong peak 1,673 cm\(^{-1}\) of the compounds might be due to the C=O stretching of –COOH group. The broadening near the base of the peak at 1,294 cm\(^{-1}\) might be due to stretching vibration of C-O of COOH group. The pattern of the aromatic C-H loop bending peaks near 693 cm\(^{-1}\) (lower intensity) and 761 cm\(^{-1}\) (higher intensity) might be due to the presence of mono-substituted benzene. From these observations, it could be concluded that the above mentioned compound might be benzoic acid. Further confirmation was carried out with the use of NMR analysis.

In compounds AC-II (1), BC-I (1), BC-II (1), CC-I (2) and CC-II (2) the presence of COOH group was evident. The peaks near 696, 808 and 896 cm\(^{-1}\) might be ascribed to the presence of meta-disubstituted benzene. A minute peak between the above mentioned peaks near 850 cm\(^{-1}\) might be due to para-disubstituted benzene. A small bulging due to merge with the base of the peak near 808 or the prominent peak at 774 [in BC-II (1)] might be due to the ortho-disubstituted benzene. The sharp peaks near 3,422 cm\(^{-1}\) might be due to the O-H stretch of –OH group. The strong peak near 1,213 cm\(^{-1}\) might be due to the C-O
stretch of OH group associated with a benzene ring. It was concluded that the structure could be a benzene ring substituted with three different functional groups in three different positions. These three functional groups are –OH and –COOH. One of them substituted the ring in two places\textsuperscript{231}. The compounds might be caffeic acid\textsuperscript{232}. Further analysis would be required for confirmation of the structure.

A similar trend of FT-IR spectra was observed for CC-II (4), BC-I (2) and CC-I (4), except the substitution pattern being prominent for a meta-di-substitution only. One of the groups was found to be OH and the other COOH. The structural elucidation would need further analysis.

The spectrum of BC-I (3) was almost similar with caffeic acid but there is a narrow and intensive band at 3430 cm\textsuperscript{-1} referring to the spectrum of ferulic acid\textsuperscript{232}.

The FT-IR spectra of BC-II (2), CC-II (3) and CC-I (3) were found to be similar with the data provided by Surowiec \textit{et al.}\textsuperscript{233}. The comparison suggested that the compound might be syringic acid. The band at 1462 cm\textsuperscript{-1} was assigned to CH stretching of methyl or methylene groups\textsuperscript{234}.

\subsection{5.5.10 Nuclear magnetic resonance}

As seen in Table 4.10 and Figure 4.22, it could be observed that all the isolated compounds could be grouped in to five different compounds. These results also coincide with the results obtained from the FT-IR analysis of 14 different fractions. Five different compounds identified with the help of FT-IR and NMR spectra were benzoic acid, caffeic acid, coumaric acid, ferulic acid and syringic acid (Table 4.10).

\subsection{5.6 Antimicrobial study}

As documented in Figure 4.23, 4.24, 4.25 and 4.26 and Table 4.11, it could be seen that the compounds were not effective against the selected fungus species but most of them were well effective against bacterial species selected for the present study. The activity of AC-I (1) and XC-I (1) was found to be the
highest against *E. coli*; and BC-I (2), CC-I (4) and CC-II (4) against *S. aeureous*. So, it could be deduced that the antimicrobial activity of 3,4-dihydroxy benzoic acid (as concluded from structure elucidation) was the highest against *E. coli*; and coumaric acid (trans-in-hydroxycinnamic acid) against *S. aeureous*. In the overall depiction it was found that *S. aeureous* (gram positive bacteria) was more sensitive to the isolated compounds as compared to *E. coli* (gram negative).

### 5.7 Compound identified

The polyphenolic compounds were purified with reference to the background that antimicrobial property might be due to their presence in the composition and was found to be true. Similar observations were reported by Agbor-Egbe and Rickard\textsuperscript{74} in aroid corms. Along with the antimicrobial property, 3,4-dihydroxybenzoic acid (protocatechuic acid) was reported to have mixed effects on normal and cancerous cells in *in vitro* and *in vivo* studies\textsuperscript{235}. Protocatechuic acid has been reported to induce apoptosis of human leukemia cells, as well as malignant HSG-1 cells taken from human oral cavities\textsuperscript{236}. Protocatechuic acid was found to have mixed effects on 12-O-tetradecanoylphorbol-13-acetate (TPA) induced mouse skin tumours. Depending on the amount of protocatechuic acid and the time before the application, it could reduce or enhance tumor growth\textsuperscript{237}. The antibacterial activity of protocatechuic acid extracted from the coffee plant was reported by Almeida *et al.*\textsuperscript{238}. The antibacterial effect of protocatechuic acid isolated from *Alchornea cordifolia* leaf was reported to be positive against *E. coli* by Lamikanra *et al.*\textsuperscript{239}. Similar activity was reported in the case of wines and many other sources\textsuperscript{240, 241, 242}. Many antibacterial and antioxidant activities of polyphenol compounds were reported by several other workers\textsuperscript{243, 244}.

### 5.8 Antioxidant activity of the pure compounds

The antioxidant activity of the pure compounds was tested using induced haemolysis by hydrogen peroxide (Figure 4.27). The haemolysis prevention was
found to be the highest in the caffeic acid and the lowest in the case of coumaric acid. The order of haemolysis was found to be coumaric acid>protocatechuic acid>syringic acid>ferulic acid>caffeic acid. The order of haemolysis prevention was found to be caffeic acid>ferulic acid>syringic acid>coumaric acid>protocatechuic acid. Out of the caffeic acids, CC-I (2) was found to have the highest haemolysis prevention, as well as to be responsible for the highest haemolysis among the group.

5.9 Starch isolation and physicochemical characterization

The starch granule size of A. paeonifolius (5-12 μm) (Table 4.12) was smaller as against the report of Hoover\(^88\) (3-30 μm). Similarly, amylose content in C. esculenta 22.4 ± 4.5% was higher than 21.4% reported by Hoover\(^88\); but, both values were lower than those reported (30.62 ± 0.16%)\(^245\). The total lipid content of C. esculenta was reported to be 0.39% against the previously reported value of 0.09 ± 0.02 (Table 4.12). The moisture content of the starch of C. esculenta and X sagittifolium was 11.2 ± 0.74 and 12.2 ± 1.2% (Table 4.12) against the earlier reported value of 14.01 ± 0.05 and 13.43 ± 0.01%, respectively\(^245\). The ash content of C. esculenta and X sagittifolium starch were 1.3 ± 0.6 and 1.2 ± 0.8% against the earlier reports of 0.31 ± 0.01 and 0.20 ± 0.04%, respectively\(^245\).

5.9.1 Starch granule morphology

In protein-starch separation, small starch granules are entrapped in the protein and the fine fiber sediments generated during centrifugation\(^156\). These problems were encountered in the case of C. esculenta and X. caracu. After the centrifugation, a dark brown layer was observed on top of the white starch. The upper layer was scraped off, but it caused loss of some small granules\(^246\). In X. sagittifolium the size of starch granules ranged from 2.1-2.84 μm which are similar to the size of 2.0–12.5 μm reported by Perez \textit{et al.}\(^245\). In the case of C. esculenta, the granule size of 0.5–5.0 μm as reported by Perez \textit{et al.}\(^245\) was much bigger to 0.71-1.25 μm, observed in the present investigation\(^245\).
5.9.2 X-ray Pattern and Crystallinity of Starch Granules

Starch is broadly divided into two types A and B; both are based on parallel standard double helixes. A-type starch helixes are more closely packed and they could be determined by X-ray diffraction studies. A-type starch (mostly cereals) exhibits reflection at 15.3, 17.0, 18.0, 20.0 and 23.4° 2θ angles. They also differ (B>A) in the content of intra-helical water. The double helices of A and B-type starches are packed in a pseudo hexagonal array. The lattice associated with B type starch has a large void (channel) which could accommodate 36 water molecules. However, in A-type starch, the lattice contains a helix in the center rather than a column of water. In both A and B-type starches, there is a spacing of double helix that corresponds to 1.10 nm distance between the axes of the two double helices. All four aroid species exhibited A-type starch pattern except an extra peak at 31.9° (2θ) in the case of *C. esculenta* (Figure 4.29). The crystallinity of starch granules was found in the order of *A. paeonifolius>* *X. caracu>* *X. sagittifolium>* *C. esculenta* (Figure 4.30). The size of crystal and the strain of polymer chain of starches were found to be in the order of *X. caracu>* *A. paeonifolius>* *X. sagittifolium>* *C. esculenta* (Table 4.14) and lattice strain refers to the slight, atomic-level displacement in the structure of a material (Figure 4.31).

5.9.3 Detection of functional groups using FT-IR Spectroscopy

The wide band observed at 3,331.91 cm⁻¹ could be attributed to O-H bond stretching of the starch and its width ascribed to the formation of inter and intra-molecular hydrogen bonds which were observed maximum in *A. paeonifolius*. Similar observation was reported by Dragunski and Pawlicka. The characteristic peak between 1,019 and 1,156 cm⁻¹ attributed to C–O stretch in C-O-C bonding and the peaks near 1081 and 1,154 cm⁻¹ could be attributed to C–O stretch in C–O–H bonding. The peak near 1,154 cm⁻¹ was not very prominent in *A. paeonifolius*. The peak near 2,930 cm⁻¹ might be attributed to the asymmetric stretching of C–H, while the band near 1,644 cm⁻¹ was ascribed to the adsorbed water and the bands near 1,420 and 1,368 cm⁻¹ to the angular
deformation of C–H; the later one was prominent in *A. paeonifolius*. The FTIR spectra not only revealed the purity of the starch isolated but also the functional groups present in the starch of the aroid species.

### 5.9.4 Gelatinization parameter

As seen in Table 4.13 the gelatinization parameters of the aroid satches found to vary from species to species. The order of ΔH was found to be in *A. paeonifolius* > *C. esculenta* > *X. sagittifolium* > *X. caracu* > Starch soluble. Similarly, the order of *T_p* was *C. esculenta* > *A. paeonifolius* > *X. caracu* > *X. sagittifolium* > Starch soluble. The ΔH generated due to the thermal decomposition of the starch granules suggested the complexity of the starch copolymer.

### 5.9.5 Recording of amylose leaching by colorimetric method

The small granule-starch tend to leach more amylose out of the intact granules at temperature 50°C and above than those of larger granules (Figure 4.33). The pattern of leaching of amylose followed the normal prediction except in the use of *X. caracu* which might be attributed to less crystallized arrangement of the polysaccharide chains, a larger portion of amorphous zone leading to more accessibility to water. Larger specific surface area might also contribute to higher water absorption by B-type granules.

### 5.9.6 Acid hydrolysis

Starch granules from the aroid species were hydrolyzed for 15 days with 2.2 M HCl at 35°C and the reducing sugar was estimated as per the standard method. The amorphous region of the starch was degraded during initial days which were followed by slow degradation of the crystalline region (Figure 4.34). The difference in the extent of acid hydrolysis of starch might be attributed to granule size, interaction in relation to amorphous and crystalline regions, composition of starch in respect of phosphate content and amylose/amylopectin ratio. In B-type starch, α-1,6 branch is located mainly
in the amorphous region making it very susceptible to acid hydrolysis; whereas, in A-type starch, α-1,6 branch is located in the crystalline region making it resistant to hydrolysis by $\text{H}_3\text{O}^+$. Starch hydrolysis could be explained on the basis of granule surface area and composition. Low amylose content and large granule size starch in *A. paeonifolius* as compared to other three aroid species might be responsible for the reduced hydrolysis during the initial and later periods. In *X. sagittifolium*, a sharp exception was observed which could be due to the starch type. In *C. esculenta*, a slight depression in the rate of hydrolysis after the ninth day might be due to the presence of amylopectins. Several workers reported wide variation (9–25 days) in regard to the time period required for the degradation of the crystalline region by $\text{H}_3\text{O}^+$ depending on the source of starch$^{251,220}$. Data suggested that during the hydrolysis, only amorphous regions were degraded by $\text{H}_3\text{O}^+$. But, the aroids did not possess sharp difference in their crystallinity. Difference in the rate and extent of acid hydrolysis among starches could be attributed to granule size; interaction between starch chains; amylopectin chain length distribution; and phosphorus content$^{252,253,220}$. But, most of these factors were negated by the size of starch granule, which was prominently revealed in the present assay. Differences in hydrolysis among the aroid starches could be attributed to the interplay of granule size, phosphorus content, and total amylose content. The difference in the extent of hydrolysis among the aroids suggested the combined effect of these three factors. In *X. caracu* and *X. sagittifolium*, the difference was negated by the granule size; and the interaction of amylose-amylopectin might be responsible for the higher rate of hydrolysis in *X. caracu* starch. The extent of hydrolysis of aroid starch (Figure 4.34) was much lower than those reported in potato, cassava, yam and sweet potato, in which starch hydrolysis exceeded 70% after 12 days$^{251,214}$.

### 5.9.7 Enzymatic hydrolysis

The susceptibility of aroid starch to α-amylase enzyme was analyzed (Table 4.12). The extent of enzymatic hydrolysis followed the order as *C.*
esculenta > X. caracu > X. sagittifolium > A. paeonifolius. The difference in \textit{in vitro} digestibility of starch among the species could be attributed to the interplay of many factors such as starch source, granule size, amylose/amylopectin ratio, extent of molecular association between starch chains, degree of crystallinity and unit cell structure\textsuperscript{211}.

Starch damage was found to be in the order of \textit{A. paeonifolius} > \textit{X. sagittifolium} > \textit{C. esculenta} > \textit{X. caracu}. The damage of \textit{A. paeonifolius} was found to be two times more as compared to others in the present investigation. Normally, amylose leaching increases due to starch damage. The starch damage of 4.54\% might be a very small factor in the case of granules having 5-12 \mu m size. This could play a vital role in amylose leaching.

The moisture content of the starch of \textit{C. esculenta} and \textit{X. sagittifolium} as reported by Perez \textit{et al.}\textsuperscript{245} (14.01 ± 0.05\% and 13.43 ± 0.01\%) was slightly higher as compared to the present finding of 11.2 ± 0.74\% and 12.2 ± 1.20\%, respectively. The results showed major difference in the composition and physicochemical properties among the aroid starches. However, variations were caused due to the interplay of factors like granule size, crystallinity and phosphorus content.

5.10 Starch and polyaniline composite
5.10.1 Morphology of the polyaniline loaded starch granules

SEM micrograph of starch (Figure 4.35) revealed hexagonal granules with the average size of 2.0 \mu m, the surface being quite smooth and well defined. In the case of the starch/polyaniline (Figure 4.35 and Table 4.15) composite with the lowest concentration of aniline, the surface of the starch granules was observed to be rough than that of the pure starch. This was indicative of polymerization of aniline over the surface of the starch granules forming a layer over it. In the case of low concentration of aniline, polymerization preferably occurred in the surface of the surface of the starch granule and then in the bulk which was evident from the micrographs (Figure 4.35). With the enhancement of aniline concentration, overgrowth of polyaniline is noticed.
5.10.2 X-ray diffraction studies

Two characteristic types of X-ray patterns of starch, A and B types are based on parallel standard double helices and the major difference between them is the packing of the helices (A-type being more closely packed); and differ in the content of intra-helical water (B>A)\(^{247}\). The starch extracted from \(C. \text{esculenta}\) was found to possess A-type X-ray pattern with characteristic peaks at \(\theta = 15.3^\circ, 17.0^\circ, 18.0^\circ, 20.0^\circ\) and \(23.4^\circ\).

X-ray diffraction pattern for the pristine polyaniline (Figure 4.36) showed two major peaks at \(\theta = 20.95^\circ\) and \(26.03^\circ\) which could be ascribed to the parallel and perpendicular periodicity of polyaniline\(^{254}\). X-ray patterns for the polyaniline/starch composites showed the evolution of peaks corresponding to the polyaniline with the increase in polyaniline concentration. Composites containing 0.1 M and 0.5 M of aniline however did not provide prominent evidence of peaks ascribed to the polyaniline. There was a prominent change in the percentage of crystallinity as observed in S2 and S3.

5.10.3 UV-Visible spectroscopy

As observed in Figure 4.38, the peak in the visible region corresponding to polyaniline shifted towards UV region in the case of S3 where as in the case of S1 it became a valley. The UV region peak of the polyaniline lost its sharpness in the composites. The UV region peak broadened up in S3 and S2, but became a small valley in the case of S1. The presence of polyaniline increased the absorption intensity of the materials after about 600 nm. The UV-Vis spectroscopy suggested the presence of characteristic changes in the material as far as the absorption of UV or visible light concerned but the composites showed the UV-Vis absorption pattern of polyaniline.

5.10.4 FT-IR spectroscopy

As depicted in Figure 4.37, the characteristic peaks of polyaniline at 1,567 and 1,483 cm\(^{-1}\) could be assigned the vibration of quinoid and benzene rings, respectively. The bands at 1,300 and 1,246 cm\(^{-1}\) corresponded to C-H
stretching vibration with the aromatic conjugation. In the case of starch as shown in Figure 4.36, the characteristic peak between 1,019 and 1,156 cm\(^{-1}\) attributed C–O stretch in C-O-C bonding, and peaks near 1,081 and 1,154 cm\(^{-1}\) could be attributed to C–O stretch in C-O-H bonding. The band near 1,644 cm\(^{-1}\) was ascribed to the adsorbed water and the bands near 1,420 and 1,368 cm\(^{-1}\) to the angular deformation of C–H. As seen in Figure 4.36, the peak at 1,567 cm\(^{-1}\) of polyaniline and 1,644 cm\(^{-1}\) of starch fuses with each other and broaden the peak suggesting the presence of moisture absorption property of starch in the composite S1. The peak near 1,154 cm\(^{-1}\) attributed to the C-O stretch in C-O-H bonding was also prominent in S1 but absent in the S2, S3 and polyaniline. The absence of the peak in 1,644 cm\(^{-1}\) in S2 and S3 suggested the loss of moisture absorption capacity of the composites. The peak at 1,483 cm\(^{-1}\) representing the stretching vibration of the benzene ring became prominent from S1 to S3 suggesting increase of polyaniline property in the composites. The bands at 1,300 and 1,246 cm\(^{-1}\) corresponding to C-H stretching vibration with the aromatic conjugation were also prominent in the composites with the increasing concentration of polyaniline (S2 and S3).

5.10.5 Differential scanning calorimetry (DSC)

The gelatinization temperature of the aroid starch was found to be 75°C [Figure 4.39 (starch)]. The exothermic curve of starch was sharp as compared to polyaniline and ending of the former was the starting point of the second. The pattern of DSC curve obtained for polyaniline was the characteristic feature of the same. In the case of S1 and S2, the gelatinization temperature increased up to 150°C as compared to polyaniline and starch. In S3 two separate peaks of gelatinization were observed, one of which was similar to that of starch but the other one near 75°C. The appearance of the new peak suggested the formation of a different composite. The overall difference suggested that composites were not only different in their thermal properties from parent polymers but differences were also prominent among them.
5.10.6 Antioxidant activity

Reactions with the fixed amount of starch-polyaniline composite with the different amounts of polyaniline and the pure components viz. starch and polyaniline were carried out and the UV-Visible spectra of the solutions were recorded after 10 min. The results for the reaction of the free radical DPPH with the composite consisting of 0.5 M polyaniline (Figure 4.41) revealed a progressive decrease in the absorption band of DPPH at 517 nm with the increasing weight of the material. The antioxidant activity of a material depends on its capability to donate hydrogen for reducing DPPH and therefore, the structural conformation of the material plays a vital role in its antioxidant activity. A single unit of the emeraldine salt form of polyaniline donates one hydrogen atom and thereby eliminates DPPH free radicals which lead to the decrease in the peak intensity at 517 nm. More is the decrease in peak intensity better is the antioxidant activity. This is possible owing to the molecular structure of the emeraldine salt form of polyaniline.

Similar antioxidant activity was exhibited by the same amount (0.4 mg) of composites and pure samples. It was observed that the antioxidant property of the composites increases with the increase in the concentration of polyaniline. This could be attributed to the fact that polyaniline owing to its redox active nature is efficient in scavenging the free radical DPPH and as such with the increasing concentration of polyaniline there is a corresponding increase in the antioxidant activity of the material. Pure starch however shows a very little activity when it comes to scavenging free radicals but polyaniline in its bulk form seems to be exhibiting the best antioxidant activity. As a result the antioxidant activity of the composites is found to be better in the samples with more amount of polyaniline which is obvious because more the amounts of the polyaniline present more is the number of hydrogen atoms donated for eliminating the DPPH.

The time dependence of antioxidant activity of an antioxidant can give important information about the reaction mechanism. This was time dependence on the antioxidant activity of the composite synthesized with 1M aniline (Figure
4.42). The antioxidant activity increased linearly up to a certain time after which the rate of DPPH scavenging decreased indicating the fact that the material was used up and there was no more hydrogen atoms that could be donated for eliminating the DPPH free radical. All composites and bulk polyaniline exhibited similar behavior. However for the composites, the reaction saturation time decreased with the decrease in the concentration of the polyaniline.

A solution of DPPH on reacting with that of a substance capable of donating a hydrogen atom, gives rise to the reduced form with the loss of violet colour. Representing the DPPH radical by $Z^\cdot$ and the donor molecule by AH, the primary reaction is:

$$Z^\cdot + AH = A^\cdot + ZH \ldots \ldots \ldots (1)$$

Where, ZH is the reduced form and $A^\cdot$ is a free radical produced in the first step. The free radical ($A^\cdot$) undergoes further reaction controlling the overall stoichiometry of the reaction. The reaction (1) is therefore intended to provide the link with the reactions taking place in an oxidizing system, such as auto-oxidation of a lipid or other unsaturated substances; the DPPH molecule $Z^\cdot$ is thus intended to represent the free radicals formed in the system whose activity is to be suppressed by the molecule AH.

The mechanism of the reaction between aniline and DPPH was investigated by many researchers\textsuperscript{255, 256}. After reacting with DPPH, the emeraldine salt-form gets converted into the fully oxidized pernigraniline of polyaniline which does not possess any hydrogen atom for reducing DPPH. The saturation in the DPPH scavenging activity (Figure 4.42) could therefore be attributed to the formation of the fully oxidized pernigraniline from the emeraldine salt form leading to loss of its free radical scavenging activity.

5.10.7 Cytotoxicity by anti-haemolysis

Polyaniline inspite of its high antioxidant activity is accompanied with certain degree of cytotoxicity. The idea of synthesizing an all organic composite consisting of a biocompatible material with a redox active conjugated polymer is intended to reduce the cytotoxicity of the conjugated polymer in order to
realize its use in biomedical applications. With the view, the anti-cytotoxicity of pure and composite samples was investigated.

Hydrogen peroxide (H$_2$O$_2$), a well known pro-oxidant can harm the blood cells by causing haemolysis. The reaction of hydrogen peroxide with iron causes the formation of free radicals and other charged species (equation 2 and 3). So, RBC (erythrocytes) itself acts as a Fenton reagent and generates hydroxyl radicals (OH*) and super-oxide anions (O$_2^-$) which finally lead to the release of iron ions from the haemeprotein$^{257}$. As a result, the blood cell gets deformed due to the formation of “splenic pitting” through proteases$^{258}$.

Fe$^{2+}$ + H$_2$O$_2$ $\rightarrow$ intermediate complex $\rightarrow$ Fe$^{3+}$ + OH$^-$ + HO$^*$ $\quad\quad$ (2)

Fe$^{3+}$ + H$_2$O$_2$ $\rightarrow$ intermediate complex $\rightarrow$ Fe$^{2+}$ + O$_2^-$ + 2H$^+$ $\quad\quad$ (3)

The haemolysis prevention activity of the composites (Figure 4.43) was better than that of polyaniline in spite of its high antioxidant activity. Pure polyaniline shows a progressive decrease in the haemolysis prevention activity. This reveals the cytotoxic nature of polyaniline which in spite of being relatively low needs to be reduced. The sample S1 showed a gradual increase in the haemolysis prevention with the increase in the weight of the material. The effect of S1 in its highest dose could be estimated from the deformation of the RBC membranes as observed in H$_2$O$_2$ treated samples (Figure 4.44). A similar behavior was also observed in S2 but its activity was less than that of S1. However, in the case of S3, there was a decrease in the haemolysis prevention activity on the addition of 1.0 mg of the composite. H$_2$O$_2$ owing to its oxidizing nature has the tendency of oxidizing the emeraldine salt. As a result, the oxidizing power of H$_2$O$_2$ gets reduced and the molecule becomes less harmful to the blood cells. But the remaining polyaniline, due to whatever low cytotoxicity it possesses, harms the RBC and leads to haemolysis (Figure 4.44). As and when the weight of polyaniline was increased the haemolysis prevention activity decreased. The fact was further confirmed by the sudden drop in the haemolysis prevention activity when 1.0 mg of the composite S3 having the highest aspect ratio of polyaniline was applied to the blood in the presence of H$_2$O$_2$. The haemolysis prevention activity was found to be the best in the
composite with the least amount of polyaniline (Figure 4.44). In the case of sample S1, the aspect ratio of polyaniline to starch was quite low and as such its haemolysis prevention activity increased with the weight of the material. When the amount of polyaniline in the composites increased, the activity of the composites got reduced. Thus, it is evident that the incorporation of starch which is non-toxic in nature reduces the cytotoxicity of polyaniline.

Investigation of organic materials for biomedical applications is not new but the conducting organic polymers having redox activity and enhanced biocompatibility could lead to the development of advanced functional materials for the biomedical applications. In the present work, starch/polyaniline composite was synthesized with a view to reduce the cytotoxicity of polyaniline. The biocompatibility of starch/polyaniline composites was found to increase with the increasing starch content and it exhibited dose dependence. Antioxidant activity alone is not enough for biocompatibility, and there is always a need of investigation in a biological media like RBC. The fact became clear when the contrast was obtained from the antioxidant activity and haemolysis prevention assay. It was observed that in spite of having very high antioxidant activity; pure polyaniline was much more cytotoxic than the composites. Looking into the bioactivity of starch/polyaniline composite, it might have the potential in the field of controlled drug release and scavenging of the oxidants generated through chemotherapeutic drugs. Subsequently, they could help in neutralizing the chemotherapeutic side effects of cancer treatment tremendously.